EFFECTS OF VARIABLE MATERNAL DIET CONDITIONS ON THE REPRODUCTIVE SUCCESS AND DEVELOPMENT OF THE CALIFORNIA SEA CUCUMBER (*PARASTICHOPUS CALIFORNICUS*)

By

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Abstract

Anthropogenic and natural climate change is altering the biology and ecology of marine organisms, which can be reflected in the supply of primary production that provides food for consumers. Primary producers differ in their biochemical composition, and marine food webs are thus based on specific combinations of producers that provide key nutrients such as dietary fatty acids (FA). Some FA cannot be synthesized by marine invertebrates, and must be acquired directly from diets. Reproductive processes in marine invertebrates are often timed to correspond with seasonal patterns in primary production, such that dietary FA and other nutrients can be partitioned to eggs to provide energy for cell division and biomolecules needed for membrane development. My dissertation investigates the consequences of changing patterns in primary production by examining the effects of maternal diet on reproductive fitness of a deposit feeder, and provides information to support the management and continued captive culturing of the commercially harvested *Parastichopus californicus* (California sea cucumbers).

In chapter 1, I describe a novel live-spawning method and quantify basic reproductive parameters for *P. californicus*. Peak spawning in the Southeast AK population was about two months earlier and three times smaller than previously observed in British Columbia, Canada. Live-spawned captive females produced more viable eggs and strip-spawned females produced higher fecundity rates. These findings are relevant for the management of commercially harvested populations of *P. californicus* because they more accurately define spawning seasons, and provide a reliable method to spawn captive animals for further aquaculture development.

In chapter 2, I present the results of feeding experiments that explore the effects of two mono-specific algal feeds with different FA profiles on female reproductive output and pre-

feeding larval fitness. Females fed with the green alga *Tetraselmis* sp. had higher fecundity, but there was reduced larval survival relative to females that were fed the diatom *Thalassiosira* sp. Similar rates of larval development were recorded in both feed treatments. Significant differences were observed in the abundance of FA 20:5ω3 (EPA), 22:3ω6 (DHA), 12:0, 16:0, and 18:0 FAs in eggs and female gonads between the two feed treatments.

In chapter 3, I used field collections in Southeast AK to assess temporal patterns feeding behavior and diet, and examined tissue-specific patterns in total lipid and FA storage and utilization, in *in situ* populations of *P. californicus*. All tissue ratios (percent of each tissue relative to the total body mass) varied significantly among collection dates. Tissue and gut content total lipid content also varied significantly among collection dates, except for muscle tissue. Shell debris and terrestrial debris were abundant in all guts regardless of collection date. FA composition differed significantly among females with different gonad maturation periods in skin, viscera, and gonads, suggesting the use of lipids stored in skin and viscera for gonad development. These results further the understanding of dietary factors affecting reproductive fitness in deposit feeders by demonstrating the importance of diet and lipid storage to gonad development.

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Introduction

Climate change is altering the biology and ecology of marine organisms (Hunt and McKinnell 2006). Among the most significant effects on marine ecosystems are the shifts in abundance and community composition of primary producers, which result in fluctuations in food supplies to pelagic and benthic invertebrate consumers. For example, shifts from dominance of large diatoms to smaller microalgal species may result from changes in oceanographic conditions (Chavez et al. 2011), and could have carryover effects on marine food webs. Coastal marine ecosystems are fueled by combinations of vascular plant detritus, macroalgae, microalgae, and, to some extent, bacteria that provide nutrients (e.g., fatty acids [FA]) to consumers (Parrish 2009, Kelly and Scheibling 2012). Some important lipids cannot be synthesized by marine invertebrates and must be acquired directly from diet (Parrish 2009, 2013). However, gross biochemical composition, particularly FA composition, differs among the plant and algal species (Parrish 2009, Kelly and Scheibling 2012) that serve as the ultimate dietary sources of these key FA. Thus, changes in the taxonomic composition of primary producers may alter the relative amounts of these nutrients available to consumers.

Quality and availability of food resources affect the body condition of consumers, which in turn has consequences for reproductive fitness (Lester et al. 2007, Poorbagher et al. 2010). Reproductive processes can thus be informative in identifying the effects of food quality on marine invertebrates. In seasonally reproducing benthic species, gametogenesis and spawning cycles are often tied to seasonal patterns in phytodetritus and algal aggregate deposition, with organisms either directly allocating these resources to fuel rapid egg production or laying down energy stores for extended gamete maturation periods (Eckelbarger and Watling 1995). Lipids

and their FA from diet provisioned to eggs provide energy for cell division and membrane development (Parrish 2009) and are essential to larval survival for all marine invertebrates.

FAs are important to animal health and must be acquired through direct consumption of primary producers, trophic transfer, or by *de-novo* synthesis (Bergé and Barnathan 2005, Reppond et al. 2008). The biochemical composition of diets, and subsequent storage of dietary lipids in tissues, has been directly linked to reproductive fitness in several commercially harvested marine invertebrate groups (e.g., shrimp, scallops, and sea cucumbers; Whyte et al. 1990, Xu et al. 1994, Ying et al. 1998, David and MacDonald 2002, Xu et al. 2016). All animals have minimum dietary requirements for essential FA; if dietary deficiencies are not met, the animal is no longer able to support cellular growth and maintenance, and will eventually die (Brown et al. 1997). The Essential FA Hypothesis proposed for Arctic fishes suggests that recent climate change is driving changes in the regional pelagic primary producers, and in turn causing declines in abundance of marine fish species that are experiencing FA limitation (Litzow et al. 2006, Litz et al. 2010).

FAs that are not *de-novo* synthesized by the animal, or not synthesized at levels that meet metabolic demands of the animal, are referred to as essential FA. Essential FA tend to be polyunsaturated FA (PUFA) and monounsaturated FA (MUFA) that are almost exclusively synthesized by primary producers (Dalsgaard et al. 2003, Parrish 2013). Most animals, particularly marine mammals and commercially important invertebrates, require six primary essential FA: docosahexaenoic acid (DHA, 22:6ω3), eicosapentaenoic acid (EPA, 20:5ω3), arachidonic acid (ARA, 20:4ω6), oleic acid (OA, 18:1ω9), α- linolenic acid (ALA, 18:3ω3), and linoleic acid (LA, 18:2ω6; Parrish 2009, Kelly and Scheibling 2012). Each essential FA serves various biological roles, and some are interchangeable (Parrish 2009). Specifically, DHA, EPA,

and ARA affect growth and reproduction in almost all animals (Bell and Sargent 2003, Boelen et al. 2013, Gladyshev et al. 2013).

DHA is particularly important in marine invertebrate larval development, and affects rates of metamorphosis into juvenile stages (Navarro and Villanueva 2000, Carboni et al. 2012). EPA and ARA are precursors to prostaglandins, and elevated levels of these FA in maternal diets have been linked to increased larval survival (Hendriks et al. 2003, Ehteshami et al. 2011, Carboni et al. 2012). OA is abundant in cell membranes and, among other metabolic roles, regulates the activate protein kinase C, a protein group that signals cell division (Chajès et al. 1995). Consequently, OA could also affect early larval development and juvenile recruitment. As a result of the importance of these FA to growth and development, fluctuations in their concentrations in food resources could directly affect consumer population stability.

In the Northwest Pacific, vascular plant inputs into marine systems come primarily from seagrasses and coastal tree litter. In general, vascular plants do not contain EPA and DHA (Ackman et al. 1968), but ALA and LA as their most abundant essential FA (Dalsgaard et al. 2003). FAs from vascular plants may not be easily assimilated by marine invertebrate grazers as cellulose-rich plant detritus has limited digestibility for many species (Fonseca et al. 1994). Thus, it is unclear if declining inputs of detritus from deciduous trees (Albert and Schoen 2013) or sea grasses (Canuel et al. 2012) will affect marine food webs.

In this same region, chlorophytes (green algae) and phaeophytes (brown algae) tend to be the primary macroalgal food resources for marine invertebrates (Harley et al. 2006, Kelly and Scheibling 2012). Chlorophytes contain abundant ARA and LA, but tend to be low in EPA, similar to seagrasses and deciduous trees (Vaskovsky et al. 1996). Specifically, Prasinophytes, Cryptophytes, and Prymnesiophytes contain high concentrations of DHA. Phaeophytes are also

characterized by relatively high concentrations of EPA and ARA. Diatoms are high in EPA and OA, and low in DHA (Vaskovsky et al. 1996). The relative proportions of macroalgal and phytoplankton taxa vary regionally and seasonally, thus affecting the supply of essential FA to marine invertebrates.

I selected the California sea cucumber (*Parastichopus californicus*) as a model organism to examine the effects of maternal diet, particularly aspects of FA nutrition, on reproduction and larval fitness. *P. californicus* commonly occurs in near-shore waters around Alaska. Deposit feeders such as *P. californicus* play important ecological roles as bioturbators and influence carbon cycling at the sea floor (Yingst 1982). This species is not only a vital food resource for sea otters and coastal Native Alaskans (Kvitek et al. 1992), but also sustains a profitable commercial fishery in Southeast Alaska and Kodiak Island (Bruckner 2004). It is the dominant sea cucumber species commercially harvested in the United States and Canada, and has one of the widest distributions of any benthic species in the Northeast Pacific, ranging from Baja California, Mexico to the Aleutian Islands, AK (Zhou and Shirley 1996).

Sea cucumbers consume many types of primary producers and/or their detritus, from vascular plants to macro- and microalgae (Lopez and Levinton 1987, Hudson et al. 2004, Slater and Jeffs 2010, Sun et al. 2015). Sea cucumbers are limited in their ability to digest most macroalgae, due to high cellulose content (Yingst 1982). Deposit-feeding sea cucumbers use specialized tentacles for selective omnivory (Boos and Reich 2010, Seo et al. 2011), chemically sensing fresh phytodetritus and actively moving toward it (e.g., Hudson et al. 2004). For example, Neto et al. (2006) found that deep-sea holothurians with peltate oral tentacles respond most rapidly to phytodetritus pulses, and that FA composition reflected variations in food supply and feeding mode. FA analysis of body tissues is thus useful in examining variations in diet,

particularly for deposit-feeding species that ingest mostly unidentifiable detrital material. *P. californicus* is a deposit-feeder that ingests surface sediment, digests the labile organic fraction, and excretes the inorganic and/or indigestible material (Cameron and Fankboner 1984). It has peltate oral tentacles with cauliflower-like structures that can actively select for food particles of different sizes and general morphologies (Cameron and Fankboner 1984).

For several decades, commercial harvests of *P. californicus* have increased due to growing market value as an exotic food and as a source of pharmaceuticals (Anderson et al. 2011). However, in 2004, the Convention on International Trade in Endangered Species (CITES) listed *P. californicus* as a species of future high concern. This listing is based on a lack of data about *P. californicus* population dynamics, and the CITES subcommittee on holothurian trade decreed a priority need for biological and ecological research on *P. californicus* (Bruckner 2004).

Maturity in *P. californicus* is reached at approximately four years post-hatching, and adults spawn annually in early summer. *P. californicus* exhibits a "conveyer belt" gametogenic mode, in which egg development occurs over the course of three years in gonad tubules (Smiley and Cloney 1985, Smiley 1988). The initial formation of egg cell walls (composed of phospholipids) occurs in years 1 and 2, while the development of egg energy reserves (triglycerides) for initial larval development occurs in year 3. Spawned eggs are fertilized in the water column where embryos develop into feeding planktonic larvae. After 1 - 4 months, larvae settle to the sea floor as 1-mm juveniles (Strathmann 1978, Cameron and Fankboner 1986). Larval mortality can be high during long planktonic periods, reducing recruitment rates. The late age of maturity can also result in slow replenishment of reproductive adults to replace harvested animals, and has contributed to population crashes in other holothurian species (Anderson et al.

2011, Friedman et al. 2011). The exact causes for population changes of *P. californicus* have not been identified; predation by sea otters (Larson et al. 2013) and fishing pressure (Anderson et al. 2011, Friedman et al. 2011) have been suggested, though dietary shifts could also contribute to reduced growth and survival as well as low reproductive output (Olsen and Henderson 1989, Lester et al. 2007).

My dissertation addresses effects of maternal diet on reproductive fitness of deposit feeders, and provides information to support the management and continued captive culturing of the commercially harvested *P. californicus*. I established hatchery culture protocols for captive broodstock, including design of a novel live-spawning method that has now been implemented in aquaculture trials, and quantified basic reproductive parameters (Chapter 1). Live-spawning methods were then used for feeding experiments that explored the effects of two mono-specific algal feeds with different nutritional profiles on female reproductive output and pre-feeding larval fitness (Chapter 2). Field collections in Southeast Alaska assessed seasonal feeding behavior and diet composition, and examined patterns in total lipid and FA storage and utilization in *in situ* populations of *P. californicus* (Chapter 3).

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Chapter 1. Spawning Dynamics of the Sea Cucumber *Parastichopus californicus* (Stimpson 1857) in Southeast Alaska

Abstract

Estimates of fecundity and spawning seasons are essential to fisheries management, but can be difficult to obtain in non-aggregating broadcast spawning species such as the commercially harvested holothurian *Parastichopus californicus*. Natural spawning cues are unknown for *P. californicus*, and spawning seasons have been determined using gonad indices (GI). I examined *P. californicus* collected over three spawning events from Southeast Alaska (SEAK) to determine the timing of seasonal spawning based on GI, and then compared GI to gonad lipid content and gametogenic stage to validate GI as an indicator of gonad maturation. I developed a live-spawning method for *P. californicus*, and obtained estimates of fecundity and viable eggs using captive live-spawned animals. Peak spawning in the SEAK population was about two months earlier, and fecundity was three times lower than previously observed in British Columbia, Canada. GI was moderately correlated with gonad lipid content and egg development period, suggesting that GI alone does not accurately reflect spawn timing. Live- and strip-spawning methods yielded significantly different fecundity and viable egg estimates, with live-spawned females producing more viable eggs g^{-1} female wet weight and strip-spawned females producing higher fecundity g^{-1} female wet weight. These findings will inform management of the commercially harvested SEAK population of *P. californicus* by more accurately defining spawning seasons, and by providing a reliable method to spawn captive animals for further aquaculture development.

1.1. Introduction

Parastichopus californicus (the California sea cucumber) is the predominant species of sea cucumber commercially harvested in the United States and Canada, and has one of the widest distributions of any benthic species in the Northeast Pacific, ranging from Baja California, Mexico to the Aleutian Islands, Alaska, USA (Zhou and Shirley 1996). In contrast to other commercially-harvested cucumbers (e.g., *Cucumaria frondosa*), *P. californicus* is a deposit-feeding species that ingests surface sediment, digests the labile organic fraction, and excretes the inorganic and/or indigestible material (Cameron and Fankboner 1984). Highest densities of *P. californicus* are found at depths between 30 and 60 m, typically on gravel and vertical rock substrates (Zhou and Shirley 1996).

Life-history characteristics (i.e., slow growth and juvenile recruitment rates) of *P. californicus* leave it highly susceptible to overfishing, leading to recent efforts to develop an aquaculture industry for this species. Like other holothurians, *P. californicus* cannot be sexed externally (Cameron and Fankboner 1986). Maturity is reached at approximately 4 years post-hatching, and adults spawn annually in early summer. Spawned eggs are fertilized in the water column where embryos develop into feeding, planktonic larvae. After 1 - 4 months, larvae settle to the sea floor as 1-mm juveniles (Cameron and Fankboner 1986). Larval mortality can be high during long planktonic periods, leading to low recruitment rates, which can become even lower if fecundity declines (e.g., Tayo et al. 2000, Becker et al. 2007). The late age of maturity can also result in slow replenishment of reproductive adults to replace harvested animals.

With rising international demands for sea cucumber products, including skin and beche-de-mer (preserved muscle), *P. californicus* commercial fisheries are expanding. Recent observations by resource management and fisheries personnel suggest that individuals,

particularly those in Southeast Alaska (SEAK), are smaller in average size and less abundant in some fishing regions than in previous decades (Clark et al. 2009, Anderson et al. 2011). As a result, there is growing interest in improving the understanding of stock dynamics and constructing aquaculture programs for this species in Alaska and Washington State (USA) as well as in British Columbia (BC), Canada. Reliable estimates of spawning seasons and reproductive output for *P. californicus* are essential to the development of fishery population models and hatchery protocols to better manage this important marine resource.

Live-spawning events for *P. californicus* have rarely been observed *in situ*, and never reported before in captivity (DeVlaming et al. 1982, West 1990). As a result, spawning seasons and gonad maturation rates for *P. californicus*, like many other commercial species, are determined using gonad indices (GI; West 1990). The basic assumption of GI (estimated as the ratio of gonad to body wall weight) is that maximum values occur when gametes are fully mature, with natural spawning events occurring soon thereafter (Cameron and Fankboner 1986). Past estimates of GI suggest *P. californicus* populations in BC reproduce annually in the summer (June into July; Cameron and Fankboner 1986). However, these data were collected over three decades ago and spawn timing has not been re-evaluated to determine if it is consistent across geographic locations and/or if it has shifted over time. GI may also be affected by individual variations in body wall and/or gonad weights, which may artificially inflate or deflate seasonal estimates. Other measures of reproductive potential and their relationship to GI, including histological observations of egg development and gonad lipid content, are also lacking for *P. californicus* (and other holothurians). Estimates of spawning season based on GI need to be validated using other measures of gonad maturation. Determining spawning seasons is essential

in management decisions regarding the timing of commercial harvests and broodstock collection for aquaculture (Caddy 2004, Bruckner 2005, Anderson et al. 2011).

Reproductive output is difficult to quantify in natural populations of non-aggregating broadcast spawning marine invertebrates such as *P. californicus*, but is nonetheless a critical parameter in stock assessments and aquaculture development because it directly affects the supply of larvae and juveniles into harvested populations (Fairweather 1991). It is commonly estimated by measuring either fecundity or the number of viable eggs produced by each female using strip-spawning methods, which involves removing the gonad from a live animal and soaking it in a chemical such as dithiothreitol (DTT). DTT induces germinal vesicle breakdown (GVB), or disintegration of the coating around the egg that is naturally removed by passage through the gonoduct during live-spawning (Courtney 1927, Johnson and Johnson 1950, Smiley 1986). However, chemicals such as DTT can cause low larval growth and survival rates in echinoids (e.g., Vacquier and Mazia 1968), asteroids (e.g., Kanatani 1975), crinoids (e.g., Holland 1976), and some species of holothurians (e.g., Chen et al. 1991); many of the eggs produced through this spawning method are non-viable. Currently, the only fecundity estimates for *P. californicus* were derived from strip-spawning and are, therefore, variable and potentially inaccurate (Courtney 1927, Strathmann and Sato 1969, Cameron and Fankboner 1989).

Natural spawning cues for *P. californicus* have not been rigorously tested, limiting the ability to maintain captive broodstock, either for experimental work to quantify life-history parameters, or for development of aquaculture (Cameron and Fankboner 1986, Smiley 1988). Spawning events for most marine invertebrates are cued by combinations of environmental parameters such as photoperiod, tidal cycles, food, and temperature (Giese 1959, Ramirez 2002), with temperature being a particularly strong cue in most currently cultured holothurians species.

A major goal of my work was to develop a replicable and logistically feasible commercial-scale live-spawning method for *P. californicus*. Variations in water temperature as small as 2 °C can initiate spawning in sub-tropical and tropical echinoderms (Morgan 2000). Therefore, I focused on water temperature as a potential cue, and developed a novel live-spawning method for *P. californicus* used to derive fecundity and viable egg estimates for wild-caught animals in an aquaculture setting.

Given the importance of *P. californicus* as a fishery and potential aquaculture product, as well as the potential ecological effects of their documented declines in abundance and individual sizes, my study addressed two main objectives. I determined the timing of seasonal patterns of spawning in the SEAK population based on GI from nine time points over two annual cycles, and then compared GI to gonad lipid content and histological observations of egg development to determine whether GI presents an accurate indication of gonad maturation. Next, I formulated a live-spawning method for *P. californicus*, and then compared fecundity estimates from three consecutive spawning seasons to those obtained through strip-spawning to determine if live-spawning can be used in large-scale aquaculture of this species.

1.2. Materials and Methods

1.2.1 Collection and Maintenance

Adult specimens of *Parastichopus californicus* (length > 10 cm; total wet mass ≥ 120 g) were hand-collected by the Southeast Alaska Regional Dive Fisheries Association commercial divers in George's Inlet, SEAK (55° 20' N, 131° 28' W) at depths of 5 - 10 m. Animals were selected from a single collection in April 2011 for use in live-spawning methods development. Additional collections for all analyses occurred every two to three months from February 2012 -

July 2013, for a total of nine collections. Live animals were placed in groups of five into plastic bags filled with seawater (7 - 8 °C), and transported in coolers via air cargo to the University of Alaska Fairbanks, Seward Marine Center. Animals typically arrived in Seward within 15 h of collection. On arrival, live animals were transferred to flow-through seawater aquaria (0.75 m x 0.75 m x 1.25 m) at 6 °C. Evisceration rates (expelling of internal organs, sometimes including gonads) were low (only 2 - 10 % of shipped animals). Although evisceration does not kill *P. californicus* (Fankboner and Cameron 1985), all eviscerated animals were excluded from my study to avoid confounding results with the effects of tissue loss and regeneration.

1.2.2 Gonad Index, Lipid Content, and Egg Developmental Stage

To estimate the timing of natural spawning in the study population, I calculated gonad index (GI; $n = 65$) and measured lipid content ($n = 60$) in most of the female gonads. Five females collected in February 2012 were not sampled for lipid analysis because at the time of collection, lipid analysis was not part of the scope of the project. Histological observations to determine egg developmental periods during peak GI were conducted on a subset of these same animals ($n = 28$). Gonads selected for histology represent the full range of GI observed, and were collected in April, June, and August 2012, as well as April 2013. Histological samples were not collected for November - February, when GIs were lowest, because too little gonad tissue was present to conduct both histology and lipid analyses on the same animal. Samples that were collected for lipid analysis and/or histological observations had one third of each gonad removed and preserved in 10 % phosphate-buffered formalin (Fisher Thermo Scientific™) for histology, and the remaining two-thirds were frozen at -40 °C for lipid analysis. For each of the nine collections, animals were sacrificed until 5-10 females were obtained because there is no

external sexual dimorphism in the species. Gonad and body wall (dermis, connective tissue, and muscle) tissues were weighed wet (to the nearest 0.01 g) using a Mettler-Toledo balance (model PB3002-S). GIs were calculated as the ratio of gonad to body wall wet weight following methods by Cameron and Fankboner (1986). Large variance in GI in a given month was used as evidence of spawning season because it suggests that both gravid (high GI) and recently spawned (low GI) individuals were present in the same population.

Gonad samples for lipid content analysis (*n = 60*) were freeze-dried in a VirTis Freeze Dryer (model 52; VirTis Company) for 32 h, and then ground prior to extraction (Oliveira et al. 2006). Lipid extractions were performed using a Dionex™ Accelerated Solvent Extractor (ASE 200) using two static cycles (5 min each) at 85 °C under N_2 gas at 1500 psi, according to the method of Dodds et al. (2004). Chromatography-grade dichloromethane (Fisher Thermo Scientific™) was used as the extraction solvent, which was treated with butylated hydroxytoluene (Sigma Chemical) at a concentration of 100 mg L^{-1} to prevent lipid oxidation. Before extraction, about 0.5 g of dry gonad was weighed on a Mettler-Toledo balance (model AE163), and then mixed with 1.0 g of Chem-tube hydromatrix drying agent (Varian INC). Gonad lipid extracts were concentrated under N_2 in a TurboVap® LV solvent evaporator (Zymark INC), incubated at 36 °C for 2 h, and then weighed again to gravimetrically calculate mg lipid g^{-1} wet gonad. Lipid g^{-1} wet gonad, instead of g^{-1} dry gonad, was used since GI also used wet gonad weights.

Prior to histological analysis, preserved gonads (*n = 28*) were dehydrated in Neoclear (HARLECO™). Full strands of gonad tubules were embedded into paraffin wax (Type 6; Fisher Thermo Scientific™). Eight 5-µm wax sections were cut from different regions of the sample using a microtome (model 820 Mark II; Reicheret-Jung), placed onto glass slides, and then dried for 24 h in a Tissue-Tek® II slide dryer (Sakura). Sections were stained with eosin Y solution

(1 % alcohol; HARLECO™) and modified Harris hematoxylin (Fisher Thermo Scientific™), according to methods by Galigher and Kozloff (1971). Twenty-five individual tubule cross sections were examined for each female, following the protocol of Foglietta et al. (2004). Five stages of oogenesis were identified in histological sections: post-spawning (PS), recovery (R), growth (G), advanced growth (AG), and mature (M; Figure 1.1.; Foglietta et al. 2004). The proportion of eggs within tubules at a given developmental stage was determined for each female, and then used to calculate the individual weighted maturity index (IWMI) using the formula below (Foglietta et al. 2004). IWMI can range from 1.00 (100 % PS tubules) to 5.00 (100 % M tubules).

$$IWMI = [\ 1(\ \%\ PS\ ) + 2\ (\ \%\ R\ ) + 3\ (\ \%\ G\ ) + 4\ (\ \%\ AG\ ) + 5\ (\ \%\ M\ )\ ]$$

1.2.3 Spawning Methods

Two spawning methods were used to estimate fecundity and viable eggs: strip-spawning and a novel live-spawning approach. For each method the ratio of male to female animals were noted following spawning methods. Previous studies suggested the annual peak spawning period for *P. californicus* is June - July (Cameron and Fankboner 1989). Thus, I selected a subset of animals from my collections in this time frame (April 2011, April 2012, June 2012, April 2013, June 2013, and July 2013) to be subjected to spawning trials. Animals were segregated in separate aquaria according to collection date. Between 45 and 65 animals were selected from each collection point, and then about 25 individuals of unknown sex were randomly assigned to aquaria (2 m x 1 m x 1 m). Stocking densities were within natural ranges observed in SEAK (Zhou and Shirley 1996). Depending on collection date, animals were maintained for 2 days to

12 weeks prior to treatments (conducted in June – July each year). Water temperatures in aquaria were slowly raised from 8 °C in April to 12 °C in July to mimic natural conditions in SEAK (Weingartner et al. 2009). Aquaria were filled with 20-µm filtered flow-through seawater (6 liters $min.^{-1}$) and lined with a 4-cm layer of sand (grain size 500 µm) to facilitate deposit-feeding. Every five days, aquaria were supplied with 10 g dry feed consisting of equal parts AlgaeMac Protein Plus, AlgaeMac 3050, and Spirulina (Bio-Marine, INC.) that was emulsified in seawater. During feeding, water flow in each holding tank was stopped for 4 h to allow emulsified feeds to settle onto the bottom of tank.

Animals for strip-spawning were collected in June 2013 ($n = 9$ females, $n = 5$ males) and July 2013 ($n = 11$ female, $n = 5$ male) and acclimated in aquaria for 4 days prior to spawning. The strip-spawning method of Maruyama (1980) was followed with minor modifications. Filtered seawater (20 µm, heated to 12 °C) was used throughout the treatment. Gonads were removed from male and female animals, and female GIs were determined as previously described. Female gonads were placed into separate containers with 850 ml of seawater. Gonads of males and females were then cut into approximately 3-mm pieces to allow sperm to be released and eggs to leak out of tubules. To induce GVB, 5 ml of 1M DTT was added to each container. After 20 min, eggs were washed through a 47-µm screen to remove the DTT solution and any remaining fragments of gonad tissue. Eggs were re-suspended in 950 ml of seawater. Sperm was pooled from five animals obtained during the same collection as females, suspended in seawater, and added to the egg suspensions at a concentration of 10,000,000 cells L^{-1}. Sperm-egg contact was allowed for 20 min for fertilization, and then fecundity, viable eggs, and egg size estimates for each female were determined as described in section 1.2.4.

Live-spawning treatments involved subjecting animals to temperature shock by transferring them from holding aquaria (12 °C) into separate spawning aquaria maintained at 18 °C, containing still seawater filtered at 20 µm and stocked with live marine microalgae (*Isochrysis* spp.) at a concentration of 10,000 cells ml^{-1} to simulate a food pulse. Four sterilized spawning aquaria (0.75 m x 0.75 m x 1.25 m) were used during live-spawning treatments. Between 10 and 25 animals were placed into each aquarium per spawning attempt. To decrease chances of polyspermy (multiple sperm fertilizing one egg, resulting in zygote mortality), males were removed from treatments once they began to spawn. Any sperm that had been released remained in the spawning aquaria to fertilize eggs. Spawned eggs were aerated in the spawning aquaria for 1 - 1.5 h to allow adequate time for fertilization to occur (Figure 1.1A). Fertilized gametes were then rinsed over 710-µm screens that retained gonad tissue and fecal matter, and a 47-µm screen that retained spawned eggs. Eggs were re-suspended in 5 L of 20-µm filtered seawater at 16 °C, and then fecundity, viable eggs, and egg size were determined, when possible, from subsamples of this suspension as described in section 1.2.4. Total wet weights were determined for each spawned female once spawning had ceased.

1.2.4 Fecundity, Viable Eggs, and Egg Sizes

For all measurements, a subsample of eggs from each female or spawning aquarium was placed on three 1-ml well slides and examined under a Leica DM2000 compound microscope. Fecundity was calculated by multiplying the average number of eggs from the three subsamples by the total volume (5 L) of the egg suspension. The total number of eggs g^{-1} spawned female wet weight in each treatment aquarium was also recorded. Numbers of viable eggs were calculated by taking the fecundity estimates and multiplying them by the percent fertilization per

female, and then recorded as viable eggs g^{-1} female wet weight in that treatment aquarium. Percent fertilization was estimated as the proportion of fertilized eggs in each subsample with no nucleus or nucleolus, denoting a zygote formation (Figure 1.1B). Egg size was determined for live-spawned females in 2011 and 2013, as well as all strip-spawned females. From each sampled female, 50 fertilized eggs (Figure 1.2) were examined prior to initial cell division with a digital camera attachment to the compound microscope, and then measured using Image J software©.

1.2.5 Statistical Analysis

All analyses were conducted using R software. Residual and normal Q-Q plots were performed prior to statistical testing. If data violated assumptions of normality and /or linearity, data were transformed accordingly. Significance levels for all tests were set at $\alpha = 0.05$. First, three ANOVAs were performed separately on log (x+1) transformed body wall weight, gonad weight, and GI data with the factors of years, months, and months nested into years to determine annual spawning seasons. Second, GI was compared with other measures of gonad maturation (i.e., lipid gonad and IWMI) using spearman correlations between non-transformed variables. Third, non-transformed estimates of fecundity and viable eggs were each compared among sampling years (2011, 2012, and 2013) using separate one-way ANOVAs to determine inter-annual variation. Non-transformed egg sizes were combined between 2011 and 2013 live spawned data, and Students' t-tests were used to determine whether egg size data differed between live- and strip-spawned females. Lastly, live-spawning year data were combined by fecundity and viable eggs estimates, and Students' t-tests were used to determine whether $x^{1/3}$ transformed data differed between spawning methods (live-spawning and strip-spawning).

1.3. Results

1.3.1 Gonad Indices (GI): Reproductive Timing

Female mean body wall weight (*n = 65*) was 122.24 ± 28.38 g (SD) (range: 60.70 – 214.16 g) and gonad weight (*n = 65*) was 4.88 ± 5.79 g (range: 0.05 – 26.30 g). Mean calculated GI (*n = 65*) was 6.31 ± 4.73 (range: 0.72 - 14.89; Figure 1.3). Body wall weight was significantly different between years (ANOVA; $F = 542.38$, $p \leq 0.001$), months (ANOVA; $F = 82.15$, $p \leq 0.001$), and months nested within years (ANOVA; $F = 6.04$, $p = 0.017$). Gonad weight did not differ among years (ANOVA; $F = 3.92$, $p = 0.053$) or months (ANOVA; $F = 1.94$, $p = 0.461$), but significantly differed among months nested in years (ANOVA; $F = 6.04$, $p = 0.017$). GI showed the same patterns as gonad weight, with no difference among years (ANOVA; $F = 1.443$, $p = 0.235$) or months (ANOVA; $F = 1.65$, $p = 0.685$), but a significant effect among months nested in years (ANOVA; $F = 5.49$, $p = 0.023$; Figure 1.3). Post hoc pairwise testing was not conducted due to small sample sizes within some collection periods. In 2012, variance in GI indicated peak spawning occurred from April (var = 12.20 GI) to June (var = 16.91 GI). In 2013, peak spawning occurred in June (var = 14.91 GI).

1.3.2 Measures of Gonad Maturation

Mean gonad lipid (*n = 60*) was 0.041 ± 0.029 mg lipid g^{-1} wet gonad tissue weight (range: 0.002 – 0.118 mg lipid g^{-1} dry tissue weight). Data shown in Figure 1.3 are presented as mg lipid g^{-1} wet weight of gonad in order to be comparable to other data presented. An individual weighted maturity index (IWMI) analysis (*n = 28*) indicated that none of the samples contained mainly post-spawned tubules (IWMI range of 1.00 – 2.00). Mean IWMI was 3.44 ± 0.69 (range: 2.24 - 4.60; Figure 1.3). GI was moderately correlated with IWMI ($r_s = 0.47$, $p =$

0.011) and gonad lipid ($r_s = 0.43$, $p = 0.024$). However, IWMI was not significantly correlated with gonad lipid ($r_s = 0.22$, $p = 0.271$).

1.3.3 Fecundity, Viable Eggs, and Egg Size: Spawning Methods Comparison

A mean of 68 % of collected animals spawned at least once in captivity (215 total collected animals for this study); however, only males spawned multiple times in different spawning trials. Prior to spawning, animals displayed "cobra" behavior, swaying with arched bodies and open oral tentacles. Males spawned first 1 h after placement into spawning aquaria. Females spawned 1.5 h after placement into spawning aquaria, usually after several males had already begun to spawn. Males and females spawned for 0.5 - 3 h with relatively constant gamete release when undisturbed.

In 2011, live-spawned fecundity was 449,588 ± 207,875 eggs female^{-1}, while viable eggs were 345,388 ± 253,113 eggs female^{-1} (*n = 18*). In 2012, live-spawned fecundity was 333,996 ± 150,758 eggs female^{-1}, while viable eggs were 317,943 ± 145,978 eggs female^{-1} (*n = 28*). In 2013, live-spawned fecundity was 279,438 ± 170,013 eggs per female, while viable eggs were 269,438 ± 173,301 eggs female^{-1} (*n = 14*). Neither fecundity g^{-1} female wet weight (ANOVA; $F = 2.145$, $p = 0.140$) nor viable eggs g^{-1} female wet weight (ANOVA; $F = 0.441$, $p = 0.649$) varied significantly between years of the live-spawning method (Figure 1.4). Pooled across years, live-spawned mean fecundity g^{-1} female wet weight was 2,863 ± 803, while mean viable eggs g^{-1} female wet weight were 2,495 ± 386. Mean strip-spawned fecundity was 382,749 ± 814,470 eggs female^{-1}, while mean viable eggs was 256,138 ± 551,793 eggs female^{-1} (*n = 20*). Strip-spawned female fecundity was highly correlated with their corresponding GI ($r_s = 0.820$, $p \leq 0.001$). Fecundity g^{-1} female wet weight ($t = 9.34$, $p = 0.004$) as well as viable eggs g^{-1} female

wet weight ($t = 14.38$, $p \leq 0.001$) differed significantly between spawning methods, with live-spawned females producing more viable eggs g^{-1} female wet weight and strip-spawned females producing higher fecundity g^{-1} female wet weight (Figure 1.4). Live-spawned eggs pooled between 2011 and 2013 had a mean diameter of 169.15 ± 19.15 µm, while strip-spawned eggs had a mean diameter of mean 146 ± 10.64 µm. Egg sizes significantly differed between spawning methods ($F = 1.22$, $p \leq 0.001$).

1.4. Discussion

Life-history parameters, including timing of spawning, sex ratios, fecundity, and gametogenesis, are important to the understanding of marine invertebrate population dynamics. In this study, I observed peak spawning for *P. californicus* in SEAK from April to June, about two months earlier than previously observed in BC. I also found that GIs were smaller in SEAK than were observed in BC in the past, indicating that reproductive potential may vary between geographic locations and/or over time. Next, GI was determined to be moderately correlated with gonad lipid and egg development stage. As part of this study, I also developed a novel live-spawning method. Although most evident in 2011, live-spawning methods from each of the three years yielded significantly higher estimates of fecundity and viable eggs than strip-spawned animals, demonstrating that my novel live-spawning method is both feasible and an improvement to currently available strip-spawning methods. With the rising international demand for sea cucumber products, my data should directly aid in management and recovery efforts for the SEAK sea cucumber fishery, and support development of aquaculture programs for this species in the Northeast Pacific.

1.4.1 Reproduction in Southeast Alaska

Current US fishery management guidelines for *P. californicus* (Clark et al. 2009) use reproductive information partially based on data collected in BC by Cameron and Fankboner (1986). My results show that these earlier data may not be applicable to populations farther north in SEAK, where peak spawning occurred from April to June, compared to June to July in BC (Cameron and Fankboner 1986). Since these populations are only separated by roughly 110 km (1.35° of latitude), differences in the observed timing of spawning are most likely due to changes in the Gulf of Alaska oceanographic conditions over time (Stabeno et al. 2004, Royer and Grosch 2006), versus latitudinal variations in reproductive potential. Seawater temperature affects adult growth and egg development rate, and cues spawning in other holothurian species (Brockington and Clarke 2001, Lester et al. 2007). Peak GI in SEAK corresponded to water temperatures of 8 – 12 °C between April and June (using monthly means from 2012 and 2013; Figure 1.1). Similar trends were recorded by Cameron and Fankboner (1986) where peak GI in BC corresponded to water temperatures of 10 – 14 °C between June and July (calculated using monthly means from 1978 to 1980). Earlier peak warm seawater temperatures could partially be due to a mean increase in seawater temperatures of 0.12 – 0.25 °C decade^{-1} in the Gulf of Alaska (Cheung et al. 2015), which could have caused the observed difference in the timing of peak GI between my SEAK and the BC studies.

In addition to differences in the timing of spawning, the values of GI also differed between the SEAK and BC regions. Peak female GI reached 28 – 32 in BC in the early 1980s (Cameron and Fankboner 1986), about 2 - 3 times higher than values observed here in SEAK (14 – 16; Figure 1.3). Lower GI indicates that SEAK body wall weights are comparatively higher and/or SEAK gonad weights are comparatively lower. Cameron and Fankboner (1986) did not

provide body wall and gonad weights separately for corresponding female GI data. However, a previous study by Fankboner and Cameron (1985) found that mean body wall weights for males and females from BC in June and July ranged from 300 - 360 g. In SEAK, female mean body wall weights during peak GI were much lower (roughly 134 g and 114 g in April and June 2012, respectively, and 108 g and 113 g in April and June 2013, respectively). Since GI is a ratio of gonad to body-wall weight, if GI and body-wall weight are both smaller, then gonad weights must also be smaller. Therefore, the SEAK population today most likely has smaller gonads, and possibly lower fecundity, than the BC population from the early 1980s.

The Gulf of Alaska has experienced decadal shifts in oceanographic conditions in conjunction with "cold" and "warm" years (Stabeno et al. 2004, Sturdevant et al. 2012). During "warm" phases of the Pacific Decadal Oscillation (PDO) the Gulf of Alaska seawater temperatures are warmer along the coast than during neutral PDO phases, there is a deepening of the mixed layer depth, and often an increase in overall production; the opposite is generally true for a "cold" phase PDO (Stabeno et al. 2004). Such shifts in regional oceanographic conditions have been linked to fluctuations in population size and reproductive potential of other Alaskan fisheries, including several species of large crab (Zheng and Kruse 2006) and most salmon species (Hare et al. 1999). The study on *P. californicus* in BC by Cameron and Fankboner (1986) occurred in 1982 – 1983 during a strong "warm" PDO phase (Glynn 1988, Masson and Cummins 2007). My study in SEAK occurred from 2012 – 2013 during a weak "cold" PDO phase (Pozo Buil and Di Lorenzo 2015). Warmer seawater temperatures and a corresponding increase in primary production in 1982-83 (Gregg et al. 2003) could have resulted in larger bodied *P. californicus* females due to the potential increase in food availability.

Variations in GI as well as gonad and body wall weights may also be due in part to fishing and/or sea otter predation pressure, which have been linked to decreases in reproductive potential in other marine invertebrates since larger sized/weighted animals tend to be targeted (e.g., Anderson et al. 2011, Purcell et al. 2014). Both SEAK and BC commercial sea cucumber dive fisheries have been operated since the mid-1980s; therefore, collections in BC occurred prior to strong fishing pressures while SEAK populations have been fished for over three decades (Bruckner 2005, Anderson et al. 2011). There is some evidence of fishing selection on SEAK *P. californicus* populations, since body wall weights were 3 times lower than those from BC. Other studies have also linked higher fishing pressure to a decrease in female abundance, mainly in species with larger females, causing shifts in female:male sex ratios from 1:1 to between 1:2 and 1:3 in exploited holothurian populations (Hasan 2005, Omar et al. 2013). I also recorded a lower ratio of females to males (1:2) in this study, in contrast to the 1:1 ratios reported by Cameron and Fankboner (1986) in BC.

Regardless of the cause, the observed differences in body wall weights and sex ratios between SEAK and BC may indicate lower reproductive potential in SEAK due to smaller body sizes and lower GI. Alternatively, if the lower GIs I observed are due to lower gonad weights, this could be due to lower fecundity and/or production of smaller diameter eggs, neither of which necessarily impacts the quality of offspring (Moran and McAlister 2009). Past studies on strip-spawned *P. californicus* from BC reported mean egg diameters of 180 - 200 µm (Smiley and Cloney 1985, Cameron and Fankboner 1986). Here, live-spawned eggs were slightly smaller (mean 165 – 175 µm dependent on spawning year), and strip-spawned eggs were much smaller (mean 146 µm). Nonetheless, updated research on the BC populations is needed to determine if

the quantity and biochemical composition of eggs truly differ between the SEAK and BC populations.

While several studies have quantified lipids from gonads of deep-sea holothurians (e.g., Hudson et al. 2004, Neto et al. 2006, Drazen et al. 2008), there are limited biochemistry data from gonads of shallow-water temperate holothurians. David and MacDonald (2002) quantified female gonad lipid content in a commercially harvested North Atlantic species (*Cucumaria frondosa,* annual mean lipid of 180 ± 40 mg g^{-1} dry weight). Female *P. californicus* gonad lipid was much lower (annual mean lipid of 97 ± 67 mg g^{-1} dry weight) and was moderately correlated with corresponding GI. Feeding ecology most likely plays an important role in the observed differences in gonad lipids between the species. *Cucumaria frondosa* are filter feeders on phytoplankton, which tends to have higher lipid content than the phytodetritus that deposit-feeding *P. californicus* consume. *P. californicus* female gonad lipid content was also weakly correlated with individual weighted maturity index (IWMI). Eggs provide the majority of triglyceride energy reserves and phospholipid cell membranes in early stages of larval development (Wehrtmann and Graeve 1998); therefore, sequential stages of egg development would not be expected to be correlated with increasing gonad lipid content.

Although GI is a useful indicator of spawning seasons, the moderate correlation with IWMI and gonad lipid suggests it may not be as reliable for marine invertebrates as for many fishes (DeVlaming et al. 1982, West 1990, Ebert et al. 2011). One potential issue is that many fish GI calculations use age and length measurements to adjust reproductive estimates for changes in gonad growth over the life spawn of the animals (Ebert et al. 2011). Holothurians from wild populations cannot be aged, and they tend to contract longitudinal muscles in various degrees such that length measurements are highly variable for a given individual. In addition,

use of GI assumes a linear relationship between body wall and gonad weights, and that gonads start developing at a hypothetical weight zero (West 1990). These assumptions are not realistic for most marine species. Holothurians do not begin to grow gonads until they reach a set weight, as in most other animals; however, current GI calculations used for holothurians do not take this into account (e.g., Cameron and Fankboner 1986, Muthiga et al. 2009). In addition, my data also demonstrated that body wall weight was not linearly correlated with GI or gonad weight. Other studies have also shown that GI does not directly scale with body weight in other holothurian species (Drumm and Loneragan 2005, Muthiga 2006).

To fully utilize GI as a reliable management tool to determine spawning seasons in adult *P. californicus*, that the method of calculating GI should be made comparable to what has been proposed for echinoids (Ebert et al. 2011). Specifically, GI calculations would need to include body weight at first gonad development, slope of the regression of gonad and body wall weights, and a seasonal weight scaling exponent. However, while most *P. californicus* females are reproductive at 4 years post settlement, the relationship between reproductive status and body weight has not been quantified (Cameron and Fankboner 1989, Hannah et al. 2012). These data are needed to formulate a new GI calculation that better estimates *P. californicus* spawning seasons.

1.4.2 Aquaculture Application and Development

Hatchery production of *P. californicus* has been hindered in the past by an inability to efficiently produce viable eggs from multiple females with predictable timing. As a result, production-scale aquaculture of *P. californicus* has not been attempted. To maximize GI, broodstock collection for Alaskan, and potentially BC, hatcheries should be in February - March

to correspond with late stages of egg development (Figure 1.3). This timing would also correspond to lowest internal organ masses (Fankboner and Cameron 1985). During shipment, *P. californicus* often eviscerate internal organs, sometimes including gonads, and then regrow organs from existing energy stores prior to spawning. Due to the remoteness of animal collection sites and the distance traveled to the Alaskan hatchery, evisceration rates can be high (about 10 to 20 % of broodstock). This is an important consideration since only the Alutiiq Pride Shellfish Hatchery located in Seward spawns animals in Alaska. Under Alaska state law, only Alaska-grown marine invertebrates (except oysters) can be out-planted in Alaskan waters for research purposes or used in flow-through grow-out facilities; thus, shipping considerations are important to broodstock spawning success and to furthering development of aquaculture.

To achieve my goal of developing a live-spawning method to reliably acquire viable eggs, I determined that seasonal seawater temperatures are important in the development of spawning methods and protocols to hold *P. californicus* in captivity. In SEAK, increases in GI coincided with increasing seawater temperatures from 5 °C to 8 °C (Figure 1.3). Maximum sustained seawater temperatures in SEAK were as high as 15 °C (Figure 1.3), indicating that seawater temperatures in spawning aquaria should be between 18 - 20 °C to reach a 3 - 5 °C temperature shock as suggested in the literature (e.g., Hamel et al. 1993, Morgan 2000). Peak GI correlated with seawater temperatures of 10 - 12 °C (Figure 1.3), demonstrating that acclimation temperatures for hatchery-held animals should be within this range to allow gametes to continue to develop in captivity.

During the three consecutive years of live-spawning methods development, fecundity and viable egg estimates did not significantly change between years (2011, 2012, and 2013), although there were large internal variations observed. I suggest that these differences are due to

the rates of germinal vesicle breakdown and fertilization being smaller in strip-spawned females, which uses the chemical DTT to remove the outer egg covering and allow for fertilization. The acquisition of fewer eggs combined with the potential for DTT to cause lower larval development rates with strip spawning make my new live-spawning method a vast improvement. Live-spawning methods for sea cucumbers are uncommon for temperate species but include other holothurians of the family Stichopodidae, including *Stichopus vastus* (Hu et al. 2010) and *Apostichopus japonicas* (Chen 2003, Fujiwara et al. 2010). Both of these species are also deposit feeders that produce planktotrophic larvae (Koushik and Raghunathan 2012, Sun et al. 2015). *Stichopus vastus* has about 6,960 viable eggs g^{-1} female wet weight (Hu et al. 2010), while *A. japonicas* has about 6,000 viable eggs g^{-1} female wet weight (Chen 2003). *P. californicus* produced over half as many eggs, which could make development of *P. californicus* aquaculture difficult because it will take two times more broodstock to collect and maintain compared to production models used in Japan. Nonetheless, *P. californicus* is currently the best available option for sea cucumber aquaculture in Alaska.

1.5. Conclusions

Life-history information for *P. californicus* has been limited, and must continue to be expanded to better manage fisheries and support aquaculture production. Although I have made several important contributions to current knowledge, there are still large gaps in the understanding of *P. californicus* life history, including natural recruitment, mortality, and predation rates. Research on juvenile ecology that would identify *in situ* "nursery" areas and environmental requirements for juvenile settlement (i.e., substrate type, food, temperature) is most urgently needed to support fisheries and hatcheries. These data gaps will need to be filled

if the commercial aquaculture production of *P. californicus* is to continue, and for existing fisheries to be effectively managed.

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1.7. Figures

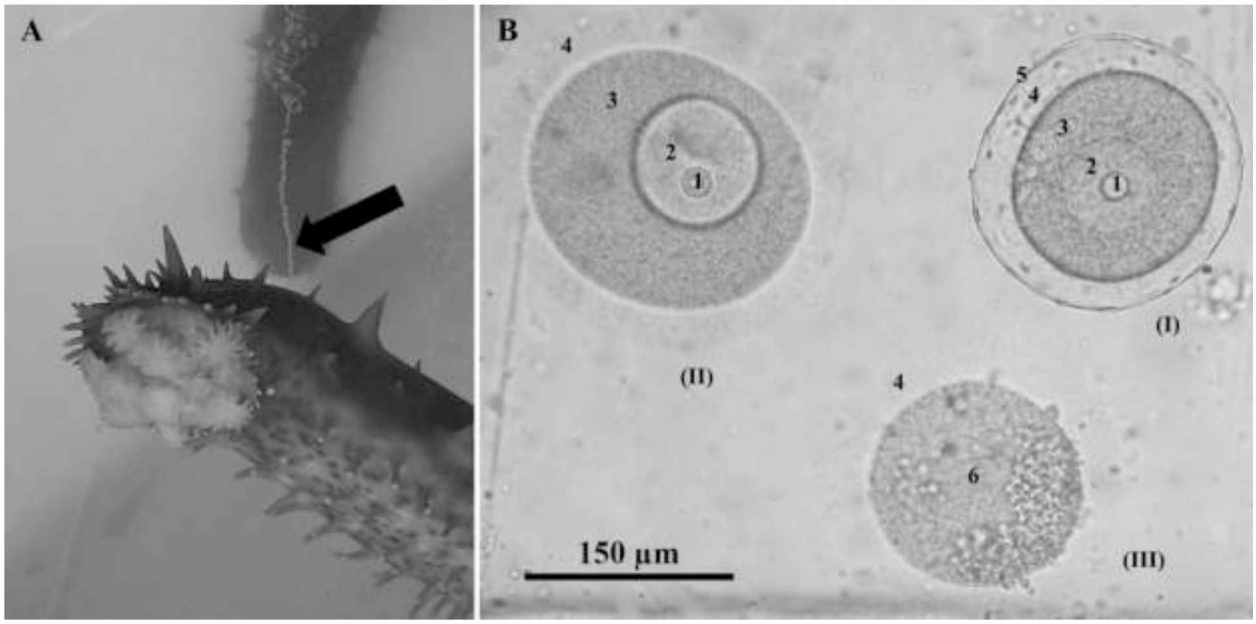

Figure 1.1. Induced spawning event and stages of egg development. (A) Induced spawning event from a female *P. californicus*, black arrow denotes egg stream from dorsal gonoduct. (B) Stages of egg development (following definitions in Strathmann and Sato 1969), 5 - germinal vesicle (GV), 4 - jelly coat, 3 - yolk, 2 - nucleus, 1 - nucleolus, 6 - zygote; (I) unfertilized egg with GV, (II) unfertilized egg without GV, (III) fertilized egg.

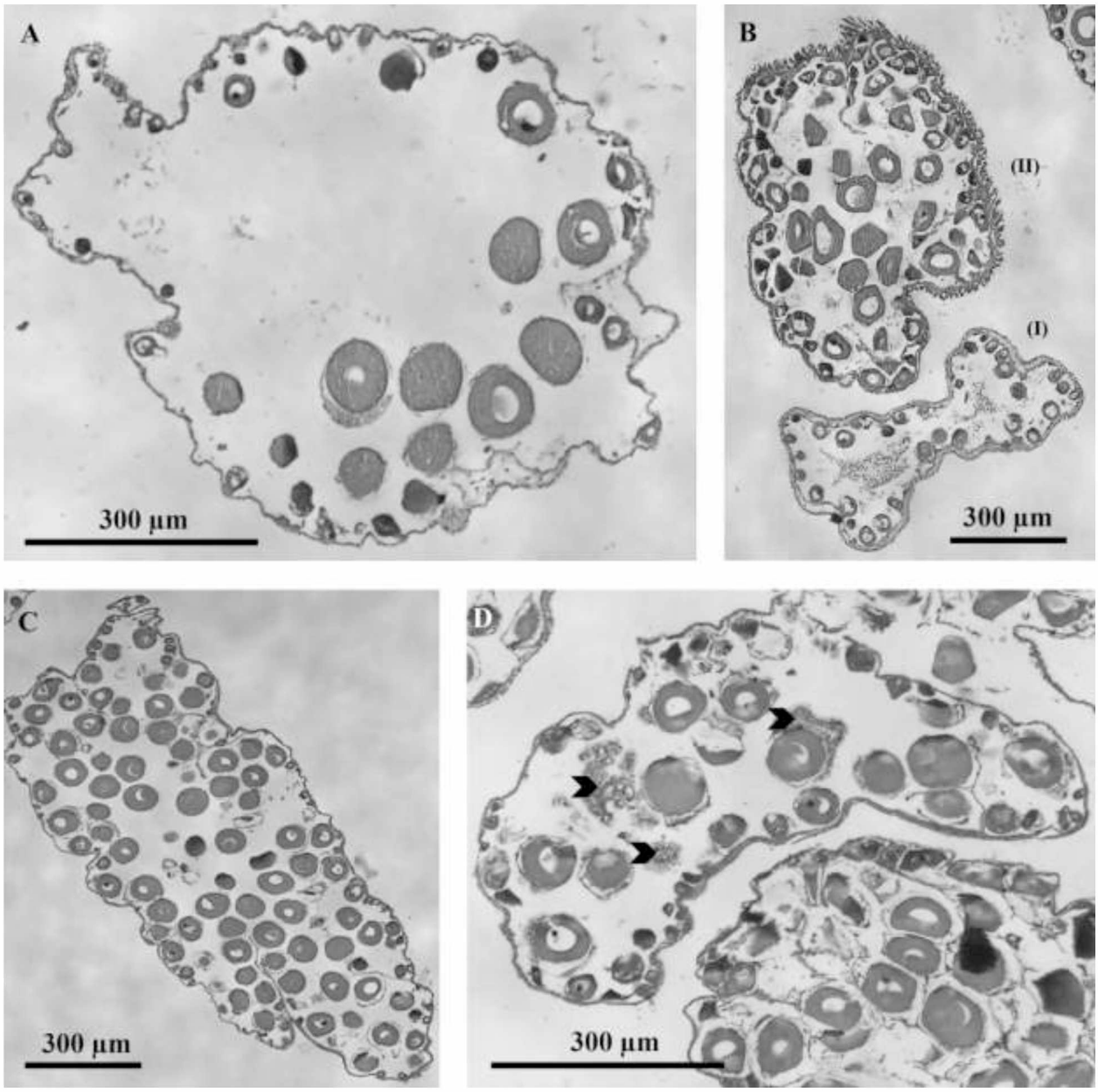

Figure 1.2. Histological analysis of tubule development (following definitions in Foglietta et al. 2004): (A) post-spawning tubules have thin walls and few mature eggs; (B I) Advanced-growth stage tubules with thick walls, and mature and developing eggs; (B II) Growth-stage tubules have thick walls and developing eggs; (C) Mature tubules full of mature eggs; (D) Recovery tubules have nutritive phagocytes denoted by black arrows.

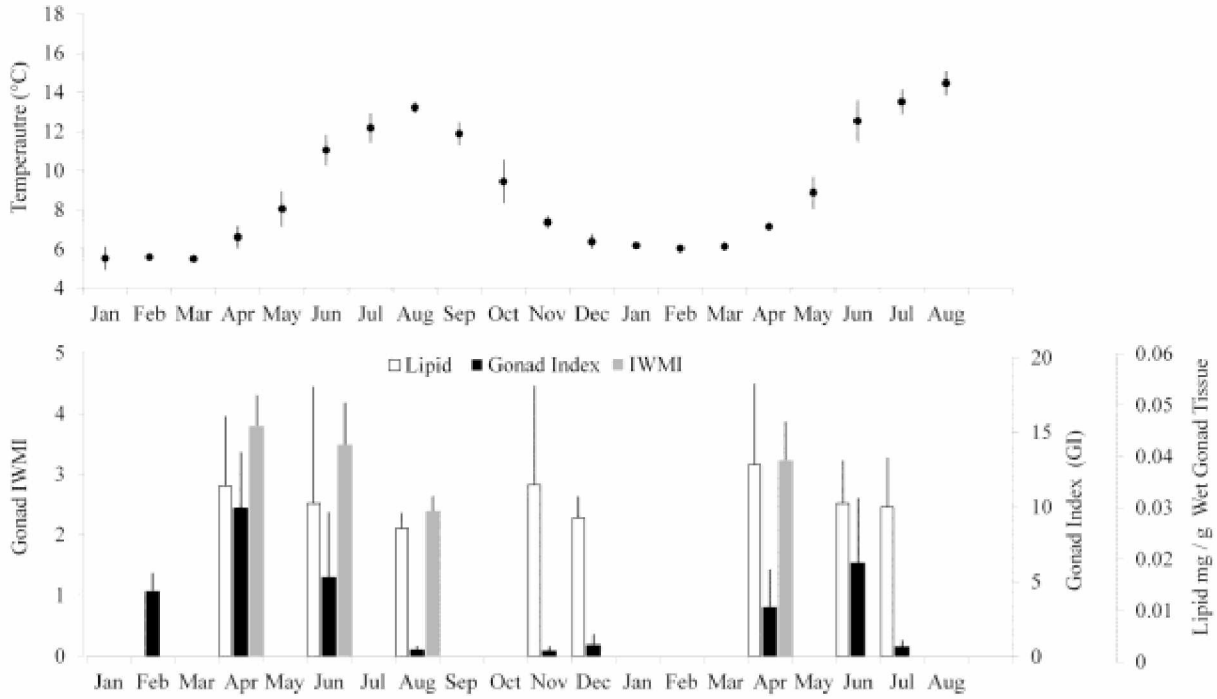

Figure 1.3. Mean monthly seawater temperatures in 2012 - 2013 from Ketchikan harbor. Data shown in °C and with ± standard deviation, and taken from a depth of 3.5 m (National Oceanographic and Atmospheric Administration buoy (Station KECA2: NOAA ID 9450460) located at 55° 19' N, 131° 37' W). Mean GI, IWMI, and gonad lipid ± SD by month and year; each variable is denoted with a separate y-axis scale.

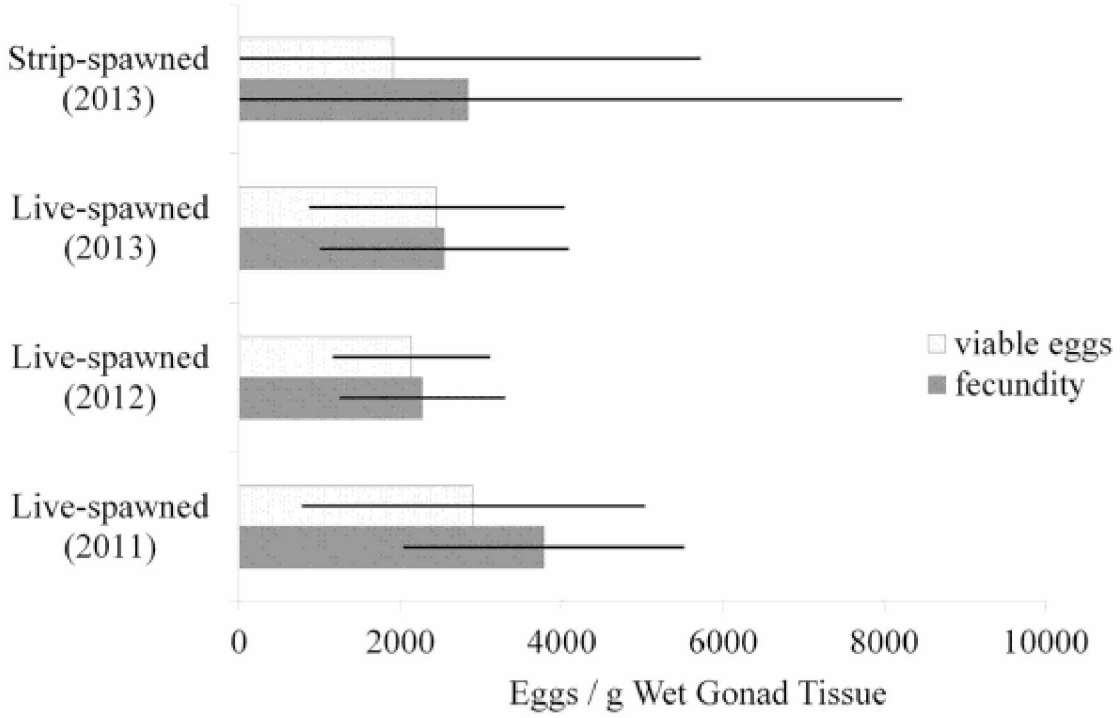

Figure 1.4. Mean total fecundity and viable eggs per g female wet weight. Data shows each year in which live-spawning was conducted (2011, 2012, and 2013), and for strip-spawning, ± standard deviation.

Chapter 2. Nutrition and Reproduction in the California Sea Cucumber *Parastichopus californicus* (Stimpson 1857)

Abstract

Phytoplankton are important sources of dietary FAs for higher trophic levels, yet FA composition of algal assemblages varies with algal species composition and growth conditions. Shifts in phytoplankton community composition may alter fatty acid (FA) availability to marine ecosystems. These shifts in the nutritional potential of phytoplankton and associated detritus may have a variety of consequences. For example, reproductive processes in marine invertebrates are tightly linked to FA composition of the diet, because invertebrates must supply their eggs with the fuel to sustain early embryonic development. I examined the effects of phytodetritus composition on the reproductive fitness of a deposit-feeding sea cucumber (*Parastichopus californicus*) through captive feeding experiments. Body- and egg-condition variables were measured in females that were fed one of two diets differing in nutritional and FA composition. Subsequent timing of larval development and survival were recorded for pre- and post-feeding stages. FA analyses were also conducted on feeds, spawned gonads, and eggs to identify specific FAs allocated to reproduction. Females that were fed the green alga *Tetraselmis* sp. had higher fecundity, but showed reduced larval survival relative to females that were fed the diatom *Thalassiosira* sp. Similar rates of larval development were recorded in both treatments. Significant differences were observed in the abundance of 20:5ω3 (EPA), 22:3ω6 (DHA), 12:0, 16:0, and 18:0 FAs in eggs and gonads from females fed the two diets. Diet-related variation in fecundity and egg quality could directly affect population dynamics of *P. californicus*, and suggests that different reproductive strategies may be favored under different environmental

conditions. If feeding conditions are favorable to planktotrophic larvae, then producing a larger number of eggs with lower energy density (such as under *Tetraselmis*-fed treatments) may be beneficial. In contrast, if oceanographic conditions are unfavorable to planktotrophic larvae, then the production of a smaller number of more energy-dense eggs (such as in *Thalassiosira*-fed treatments) could result in higher overall recruitment.

2.1. Introduction

Climate variability is causing large-scale changes in patterns of primary production and species composition of marine phytoplankton (e.g., Chavez et al. 2011), and these changes may have important nutritional consequences for marine consumers (Parrish 2009, Kelly and Scheibling 2012). Phytoplankton community composition is influenced by oceanographic conditions, including nutrient and freshwater inputs and sea surface temperatures (Behrenfeld et al. 2006). For example, while the green alga *Tetraselmis* has been found in low abundances in the North Pacific over the last few decades (Hori et al. 1982, Balzano et al. 2012), large blooms of *Tetraselmis* have occurred since the early 2000s at temperate Pacific latitudes, where mean sea surface temperatures have warmed roughly 2 – 5 °C (Pizarro et al. 2012, Southern California Coastal Ocean Observing System 2013). Regional warming and freshening of surface waters favors smaller green algae and flagellates, and may stimulate future such blooms at higher latitudes in the Northwest Pacific, in areas where diatoms are now seasonally dominant (Laws et al. 1988, Morán et al. 2010, Chavez et al. 2011).

Decreased abundance of commercial fish and shellfish species such as salmon and Dungeness crabs (Hare et al. 1999, Zheng and Kruse 2006) has already been linked to declines in coastal diatom biomass in the Northeast Pacific (Chappell et al. 2013). Changes in the species

composition of phytoplankton assemblages can impact consumers because the biochemical composition of phytoplankton (and the resultant phytodetritus they produce) varies among taxa, and with environmental conditions. In particular, fatty acid (FA) composition provides a good indicator of these differences, including taxonomic effects of growth conditions (e.g., irradiance and temperature; Guschina and Harwood 2009, Leu et al. 2010), and changes in composition that occur over the course of a bloom event (Wang et al. 2014).

Declines in diatom biomass, and the subsequent shifts in phytoplankton community composition, could have direct effects on the regional food web including a decrease in essential FAs (Litzow et al. 2006, Kelly and Scheibling 2012). Diatoms are particularly high in the essential polyunsaturated fatty acid (PUFA) 20:5ω3 (eicosapentaenoic acid, EPA), but they are low in the PUFA 22:6ω3 (docosahexaenoic acid, DHA; Parrish 2013). In contrast, green algae are generally high in EPA and DHA (Dunstan et al. 1993, Dalsgaard et al. 2003, Kelly and Scheibling 2012). Although there is evidence that some species of marine fish can synthesize PUFAs *de-novo*, most marine invertebrates cannot, and only have limited ability to elongate precursors into long-chain PUFAs such as EPA and DHA (Brett and Muller-Navarra 1997, Litzow et al. 2006, Parrish 2013). Thus, many FA that are particularly important to animal health must be acquired through direct consumption of primary producers or through trophic transfer (Bergé and Barnathan 2005, Reppond et al. 2008). Consequently, changes in the species composition or growth conditions for primary producer communities may lead to changes in the supply of these essential nutrients to the food chain.

The importance of FA content in the diet of marine consumers has already been documented in marine fish and invertebrates. The Essential FA Hypothesis proposed for North Pacific fishes suggests that recent climate change is driving shifts in the relative population size

of different forage fish species as a result of differences among taxa in susceptibility to FA limitation (Litzow et al. 2006, Litz et al. 2010). In marine invertebrates, levels of essential FAs (e.g., EPA, DHA, and 20:4ω6 - arachidonic acid [ARA]) in the diet affect reproductive output, lipid density of eggs, and larval survival (e.g., Xu et al. 1994, Hendriks et al. 2003, Ehteshami et al. 2011). FAs provisioned to eggs are utilized as energy reserves (as triglycerides [TAG]), and as structural components of cell membranes in developing embryos and larvae (as phospholipids [PL]; George 1994). High levels of EPA and DHA in adult diets result in the production of larger, faster-growing larvae with better survival rates in shrimp and oysters (Xu et al. 1994, Hendriks et al. 2003). The percent composition of saturated fatty acids (SFA; specifically 14:0, 16:0, and 18:0) in phytoplankton supplied as larval feed has also been correlated with larval growth and survival rates (Goedkoop et al. 2007), possibly because energy stored in SFAs is released more quickly and efficiently than in more complex unsaturated FA. Thus, changes in the relative abundance of FAs in the diet of marine invertebrates due to regional shifts in the primary productivity regime may have consequences for reproduction and recruitment success in consumers. I used the commercially-harvested sea cucumber, *Parastichopus californicus*, to directly test the effects of changes in species composition of the algal food source on reproduction.

P. californicus has one of the widest distributions of any benthic species in the Northeast Pacific, ranging from Baja California, Mexico to the Aleutian Islands, Alaska, USA (Zhou and Shirley 1996). Deposit feeders such as *P. californicus* are bioturbators that influence carbon cycling at the sea floor (Yingst 1982). *P. californicus* is also a food resource for sea otters and bearded seals (Kvitek et al. 1992, Clark et al. 2009) and supports profitable commercial fisheries in the region. Recent reports suggest that some populations of *P. californicus* have a smaller-

than-average size and are less abundant than in previous decades (Clark et al. 2009, Anderson et al. 2011). Although exact causes of population fluctuations are unknown, shifts in phytoplankton / phytodetritus composition have been indicated for other taxa, as outlined above, and may be a factor here due to regional changes in oceanographic conditions (Strom et al. 2015).

Adult *P. californicus* spawn annually in early summer, and spawned eggs are fertilized in the water column where embryos develop into feeding planktonic larvae. After one to four months, larvae settle to the sea floor as 1-mm juveniles (Cameron and Fankboner 1986). Larval mortality can be high during long planktonic periods, leading to low recruitment rates, and decreased fecundity may further impact recruitment (e.g., Tayo et al. 2000, Becker et al. 2007). Maturity is reached at approximately four years. The late age of maturity can lead to overexploitation if new cohorts of juveniles do not replace harvested adult animals. These life-history characteristics of *P. californicus* also leave it vulnerable to population declines resulting from environmental change. Therefore, identifying factors that affect reproductive output and recruitment rates in a changing environment will support management efforts for *P. californicus* populations and other benthic deposit feeding marine invertebrates.

I conducted controlled feeding experiments with individuals collected in the Gulf of Alaska, USA, and examined the differences in body condition, reproductive output, and pre-feeding larval development in animals that were supplied with two different mono-specific algal feeds with different FA profiles: the green alga *Tetraselmis* sp., and the diatom *Thalassiosira* sp. I tested the following three hypotheses: 1) phytoplankton species composition of detrital feed provided to adults affects female body and egg condition; 2) larvae from females fed different

diets vary in their developmental and survival rates; and 3) differences in female diet are reflected in total lipid content and FA signature of eggs and gonad tissues.

2.2. Materials and Methods

2.2.1 Female Collection and Maintenance

Adult specimens of *P. californicus* (n = 120; total wet mass ≥ 95 g) were hand-collected by divers in Southeast Alaska (SEAK; 55° 20' N, 131° 28' W) on Dec. 10, 2012. Live animals were placed in groups of five in plastic bags filled with seawater (7 - 8 °C), and transported in coolers via air cargo to the University of Alaska, Fairbanks (UAF) Seward Marine Center (Seward, Alaska). Transport time was about 15 h from collection to transfer into experimental tanks, with no acclimation period. Evisceration rates (expelling of internal organs, sometimes including gonads) were low (8 % of collected animals). Although evisceration does not kill *P. californicus* (Fankboner and Cameron 1985), all eviscerated animals were excluded due to potential reduction in the gonad quality if energy reserves are mobilized from gonads during regrowth of viscera.

Adults were maintained in twelve experimental tanks (six tanks for each of the two feeding treatments) for the duration of the 32-week feeding experiment (Figure 2.1). Each 1 m x 1 m x 2.5 m tank was stocked with seven randomly selected animals, for a total of 84 animals. Sex cannot be externally determined for holothurians, but 1:1 sex ratios have been reported for this species (Cameron and Fankboner 1986), so each experimental tank was stocked with seven individuals in an effort to ensure multiple females were supplied to each tank. The stocking densities of tanks were within ranges typically found in SEAK (2 – 12 animals m^{-2}; Clark et al. 2009). The tank bottoms were lined with a 5-cm layer of sterilized sand (grain size 2 mm) to

facilitate deposit feeding, and filled with filtered (20 μm) flow-through seawater (salinity 30). Seawater temperatures and light-dark cycles were adjusted bi-weekly to simulate the ambient conditions of SEAK based on National Oceanographic and Atmospheric Administration buoy data for Ketchikan, Alaska (AK; ID 9450460; URL: http://pajk.arh.noaa.gov/).

2.2.2 Feed Treatments

Tanks were randomly supplied with one of two feeds: the diatom *Thalassiosira* sp. (TW), or the green alga *Tetraselmis* sp. (TS; Figure 2.1). Feeds were chosen to represent phytoplankton taxa that are favored under different oceanographic conditions in the Northeast Pacific (Jester et al. 2009, Balzano et al. 2012, Strom et al. 2015). Although natural diets would not consist of a single algal species, the selected taxa contain significant differences in FA profiles that are representative of bloom-forming species typical of the Northeast Pacific and Alaska (Viso and Marty 1993). Algal feeds were purchased as whole-cell live concentrates from Reed Mariculture Inc. The use of commercially available mono-specific algal cultures ensured uniformity among batches of feeds supplied periodically throughout the experiments.

To ensure that minimum energetic requirements were met during the experimental period, a minimum feed quantity was set at 0.369 g total organic matter (OM) animal^{-1} day^{-1}. This amount was reported by Yingst (1982) as sufficient for maintenance of the closely-related, similarly-sized species *Parastichopus parvimensis*. OM content was determined from ash-free dry weight of algal feeds by combusting 2 g of each feed in a Stybron Thermolyne™ furnace (model FA1730) at 450 °C for 36 h. Feed quantities for the treatments were then normalized to carbon (C) content in order to quantify the amount of each feed that should be supplied to approximate detrital fluxes typical of this region, commonly reported in units of mass of C area^{-1}

time^{-1} (Shimanaga et al. 2000). Percent C and nitrogen (N) of the feeds were determined using a NC 250 Carlo Erba elemental analyzer at the Alaska Stable Isotope Facility at UAF, and then percent N was by multiplied by a factor of 6.25 to obtain percent protein (P; Jones 1941). Final feed levels were set at 43.44 ml of TW m^{-2} day^{-1} and 12.88 ml of TS m^{-2} day^{-1}. Algal concentrates were frozen into equal-sized pellets, each containing 2 g of sterilized sand to ensure feeds were not suspended and washed out of tanks. Frozen pellets were then supplied to each tank twice a week and allowed to thaw, maintaining constant food availability. Sediment lipid content in experimental tanks was measured three times at regular intervals during the experiment (beginning, middle, and end; approximately 75-day intervals).

2.2.3 Induction of Spawning, Female Body Condition, and Egg Condition

In order to retrieve gametes for FA analysis and larval culture, adults from experimental treatments were induced to spawn using a newly formulated method for this species that involves a 10 °C temperature shock in combination with exposure to live *Isochrysis* sp. supplied at 10,000 cells ml^{-1} to simulate a large algae bloom (Chapter 1). Spawning induction began in May 2013 to correspond with natural reproductive cycles of *P. californicus* from SEAK (Chapter 1). Female spawning occurred over a four-week period from May through June 2013. Animals were moved to separate spawning tanks for spawning induction, with all individuals from each replicate experimental tank transferred to a single separate spawning tank. The first female to spawn was isolated by removing all other animals, leaving only the single spawning female and any spawning males in the spawning tank. Males always spawned first, and at least one male spawned in each tank prior to each female spawning. When females finished spawning, gametes were collected by draining the tank and filtering seawater onto a 47-µm mesh screen.

Fertilization occurred uncontrolled in the spawning tank, allowing multiple males to fertilize the eggs of each female.

Spawned females were dissected at the completion of spawning, and tissue weights were recorded. The following female body condition variables were quantified: g total wet weight, spawned gonad index (GI), and eggs g^{-1} female wet weight. GI is estimated as the ratio of gonad to body-wall wet weights. Not all animals spawned at once or in the same spawning treatment. Since females were dissected after they spawned, replacement animals of equal size were placed into experimental feeding tanks in order to maintain stocking densities for the remainder of the experiment. Replacement animals were tagged with green ultraviolet elastomer polymer tags (Northwest Marine Technologies Inc.). Replacement animals were not induced to spawn in subsequent spawning treatments and excluded from the final analysis.

To determine egg condition variables, three 1-ml subsamples were taken from a concentrated 10-L egg solution, and then counted under a Leica DM2000 compound microscope. Counts from subsamples were multiplied by the total volume of egg solution to calculate total fecundity. Fecundity estimates were normalized to whole-body wet weight. Egg diameters were measured for 20 eggs $female^{-1}$ prior to the onset of cell division from still images using ImageJ software (Abràmoff et al. 2004). The proportion of fertilized eggs was recorded 1 h post-spawning by examining 100 eggs. About 16,000 eggs from each spawned female were allocated to larval survival and developmental rate experiments; the remaining eggs were concentrated and frozen for future lipid and FA analyses.

2.2.4 Larval Culturing and Development

Fertilized embryos were cultured to measure effects of maternal diet on larval survival and rate of development for pre- and post-feeding larval stages (Figure 2.2). Larvae were not fed in order to test the carryover effects of maternal diets on larval starvation resistance and limit confounding effects of larval feeding on results. Developing embryos were suspended in 800-ml glass containers using a motorized paddle system, with ten replicate containers per spawned female. Stocking densities were 2 embryos ml^{-1} sea water. Containers were nested within a larger seawater bath heated to approximately 10 °C to reflect typical seawater temperatures encountered in May through July at the adult collection site in SEAK. Water changes occurred every 36 h post-fertilization with 10-µm filtered UV treated seawater. Larval survival and development were calculated by removing a 4-ml sub-sample every 36 h from each container and counting the number of live larvae at each development stage (fertilized egg, first cleavage, gastrula, auricularia, and doliolaria; Figure 2.2).

2.2.5 Lipid and Fatty Acid (FA) Analysis

Spawned gonads and eggs were analyzed for total lipid and FA content to determine whether differences between feeds affected provisioning of FAs to gonad and eggs that matured during the experimental period. Skin and muscle samples were also analyzed for total lipid content as a metric of female body condition. Samples were frozen at -40 °C until processing and then weighed wet and freeze-dried in a VirTis Freeze Dryer (model 52; The VirTis Company) for 32 h. A MettlerToledo analytical balance (model PB3002-S) was used for all mass determinations. Lipid extractions were performed on freeze-dried material using a Dionex™ Accelerated Solvent Extractor (ASE 200) operated at two 5-min static cycles, 85 °C furnace, and

under 1500 psi N_2 gas according to methods by Dodds et al. (2004). Grade 99.98 % purity dichloromethane (DCM) was used as the extraction solvent (Fisher Thermo Scientific™), which was treated with butylated hydroxytoluene (BHT; Sigma Chemical) at a concentration of 100 mg L^{-1} in DCM to prevent lipid oxidation. Lipid extracts were concentrated in a TurboVap® LV solvent evaporator (Zymark INC) under N_2 at 36 °C for 2 h, and then weighed to gravimetrically determine lipid content.

FAs were converted to FA methyl esters (FAME) using an acid-catalyzed esterification with Hilditch reagent according to Iverson et al. (2002) and quantified using a gas chromatograph coupled to a flame ionization detector (GC-FID) with identical chromatographic conditions to those described by Farrugia et al. (2015). A maximum of 20.0 mg lipid was esterified from each replicate. FAMEs were concentrated using a TurboVap® LV solvent evaporator (Zymark INC) and FAME weights were recorded prior to identification by injection into an Agilent model 6850N Series II GC-FID and fitted with a DB-23 (60 m × 0.25 mm, 0.25 µm film) capillary column (Agilent Technologies). Peak areas of 37 FAs were identified by comparing retention times to those of the external standard Supelco 189-19 FAME mixture (Sigma-Aldrich). Response factors were calculated for FAs in based on the standard 189-19 mixture, and then used to correct peak areas of FAMEs detected in samples following the method described by Ackman and Sipos (1964). Equations for the calibration curves are shown in Table A-1. Remaining FAs were identified using a GC model 6890 interfaced with a mass spectrometer (MS) detector model 5973N (Agilent Technologies) and comparing mass spectra of FAMEs to spectra in the MS Library (05 v.2.0). Sixty-seven FAs were quantified consisting of at least 95% of total peak areas in any given sample. Data were quantified as concentrations (spawned gonad: mg FA g^{-1} dry tissue; eggs: mg FA 10,000 eggs) using linear calibration curves

determined for FAs in an external standard mixture (Supelco 189-19). Egg FA data were not recorded as mg FA mg^{-1} eggs because of the difficulty of removing all of the water and associated salts from the egg mixture prior to analysis, which could have added substantial sample mass. For FAs identified in samples by GC-MS that were not present in the external standard mixture, calibration curves for FAs of same carbon chain length and number of double bonds were used (e.g., linear equation for 20:1ω9 was used to quantify 20:1ω7).

2.2.6 Statistical Analysis

A series of comparisons was made among spawned females, eggs, and larvae from the two experimental feed treatments. Experimental tanks, not individual females, were used as replicates for statistical analyses. In cases where there was more than one spawned female per tank, data from multiple females were averaged to yield a single data point for each tank. Diagnostic testing was performed prior to statistical analyses. If data violated assumptions of normality or linearity, they were transformed accordingly (i.e., survival data were square root transformation). Larval survival data were arcsine transformed because they were quantified as percentages. The significance level for all tests was set at $\alpha = 0.05$.

Univariate data were analyzed using R software (R Development Core Team 2008). Each female body and egg condition variable was compared separately between feed treatments using Student's t-tests. Bonferroni corrections were applied where multiple analyses on the same sample were performed (fecundity, diameter and fertilization rate; gonad total lipid and total FA). Next, percent larval survival was compared between treatments using repeated-measures analysis of variance (repeated-ANOVA) where survival was the repeated measure. Lastly, the concentrations of EPA, DHA, ARA, Σ SFA, and Σ PUFA in feeds, spawned gonads, and eggs

were compared between experimental treatments using separate Student's t tests with Bonferroni correction.

Multivariate FA data were analyzed using PRIMER-E software version 6 (Clarke and Gorley 2001). FA concentrations from feeds, spawned gonads, and eggs were standardized prior to analyses to account for differences in measurement units (mg FA g^{-1} tissue for feeds and gonads versus mg FA 10,000 eggs $^{-1}$). Due to the high water content in eggs, it was not possible to quantify FA in the same units as tissues and feeds. An analysis of similarity (ANOSIM) was used to determine if there were significant differences in FA composition between treatments for spawned gonads, and for spawned eggs. Subsequently, similarities percentage (SIMPER) analysis was conducted to determine which FAs contributed most to dissimilarities among feed treatments as a pairwise testing procedure. Data were then visualized using non-metric multi-dimensional scaling ordinations (nMDS).

2.3. Results

2.3.1. Adult Feed Treatments

Concentrations of all biochemical components were higher in the TS feed than in the TW feed, except for total lipid, which was lower in TS than in TW (Table 2.1). No statistical testing was conducted on feed data due to lack of replicates; however, ANOSIM showed no significant difference between feeds based on these parameters ($p = 0.010$, $R = 0.999$) and feeds were 57.55 % dissimilar in terms of FA composition (SIMPER, Table 2.3). FAs contributing most to dissimilarities between feeds included EPA, ARA, 16:1ω7, 18:3ω3, and 14:0 (Table 2.3). Concentrations of ARA and Σ PUFAs were higher in TS, while EPA, DHA, and Σ SFA were higher in TW (Figures 4 & 5). Lipid content of sediment samples collected from replicate

experimental tanks did not significantly differ between feed treatments ($F = 2.38$, $p = 0.195$; beginning: TS 2.39 ± 3.94 mg lipid g^{-1}, TW 4.84 ± 4.98 mg lipid g^{-1}; middle: TS 4.19 ± 3.36 mg lipid g^{-1}, TW 2.37 ± 2.67 mg lipid g^{-1}; end: TS 2.37 ± 2.81 mg lipid g^{-1}, TW 3.85 ± 3.01 mg lipid g^{-1}).

2.3.2. Female Body Condition and Reproductive Output

In total, 13 females from 10 of the 12 tanks successfully spawned and could be included in the study (Figure 2.1). Tanks two and six (TW) and tank one (TS; Figure 2.1) were not considered in data analyses because either none of the females spawned, or because spawned females had eviscerated. Males spawned in all experimental tanks. Sex ratios were determined following dissection of experimental animals, and did not differ significantly between feed treatments (individual Student's t-tests: died during experiment: $t = 2.34$, $p = 0.999$, unknown sex: $t = 1.12$, $p = 0.338$, males: $t = 0.86$, $p = 0.670$, spawned females: $t = 1.77$, $p = 0.734$, and eviscerated or unspawned females: $t = 1.39$, $p = 0.022$; Figure 2.1). None of the female body condition variables were significantly different between feed treatments ($p \geq 0.05$; Table 2.2).

FA composition of spawned gonads was 73.16 % dissimilar between feed treatments, but was not significantly different between treatments (ANOSIM; $p = 0.238$, $R = 0.094$). High non-significant dissimilarity between samples was most likely due to the small sample size of spawned gonads. The FAs that contributed most to dissimilarities in spawned female gonad composition between treatments included ARA, DHA, EPA, 22:4ω6, 16:0 and 16:1ω7 (Table 2.3). EPA, DHA, ARA, ΣSFA, and ΣPUFA from spawned gonads did not significantly differ between the feed treatments, but variance was high among samples (Figure 2.4 and 2.5, respectively). However, overall trends showed higher levels of ARA, EPA, DHA, Σ SFA, and Σ

PUFA in spawned gonads from females fed TS, compared with spawned gonads from females fed TW (Figure 2.4 and 2.5).

Based on observations of wild-caught specimens, females tend not to spawn all eggs within gonads (Chapter 1), and individuals often contain eggs at various developmental stages throughout the year (Smiley 1988). Estimates of GI for spawned experimental animals may provide information on reproductive potential for subsequent spawning seasons. GI were compared to data from *in situ* SEAK animals to determine how similar conditions in experimental tanks were to natural conditions at the collection site. Spawned gonads in wild-collected animals from SEAK in July 2013, the time corresponding to experimental spawning, had mean lipid of 5.31 ± 3.98 mg lipid g^{-1} wet tissue (Chapter 3.4). Mean gonad lipids for experimental treatments were not significantly different from the wild-collected females (Table 2.2; Student's t-tests: TS t = 0.12, p = 0.998; TW [t = 1.32, p = 0.996]). In SEAK, mean spawned gonad weights in July 2013 were 0.86 ± 0.47 g or a GI of 0.69 ± 0.39 % (Chapter 1). Although mean spawned gonad weights were higher in experimental females, neither treatment was significantly different from SEAK females (Table 2.2; Student's t-tests: TS t = 2.04, p = 0.18; TW t = 1.67, p = 0.110). In addition to FA, various spawned gonad metrics were used to determine differences in fecundity between feed treatments (Table 2.2).

2.3.3. Egg Condition

Egg diameter, percent fertilization, and lipid content of eggs were not significantly different between feed treatments (Table 2.3). However, fecundity was significantly different (p = 0.016). Females fed TS produced more eggs than females fed TW (2,308.43 ± 699.75 versus 1,121.38 ± 411.29, respectively; Table 2.3). FA composition of eggs was also not significantly

different between treatments (ANOSIM; $p = 0.310$, $R = 0.044$) but showed 79.70 % between group dissimilarity. High non-significant dissimilarity between samples was most likely due to the small sample size of eggs. FAs contributing most to dissimilarities in eggs between treatments included 16:0, 18:0, 12:0, and 14:0 (Table 2.3). As above, concentration of essential FA and FA classes in eggs did not significantly differ between feed treatments, but again variance was high. Trends showed higher levels of ARA, EPA, DHA, Σ SFA, and Σ PUFA in eggs from females fed TW, compared with eggs from females fed TS (Figures 2.4 and 2.5).

2.3.4. Comparisons of FA Composition among Sample Types

Specific FAs were also compared between feeds, as well as spawned gonads and eggs from each treatment (Figure 2.6 and 2.7). Elevated levels of a specific FA in spawned gonad and/or egg samples relative to the corresponding feed denoted FA enrichment in that tissue type. For each experimental treatment, FA composition significantly differed between the feed, gonads, and eggs produced in that treatment (TS: $p = 0.001$, $R = 0.884$; TW: $p = 0.001$, $R = 0.948$). In both treatments, eggs were higher than feeds in 12:0, 14:0, 14:1ω3, 16:0, and 18:0. Additionally, eggs from females fed TW had higher 24:0, and eggs from females fed TS had higher ARA (Figure 2.6). Eggs from the TW treatment were lower than feeds in 15:0, 16:1ω7, 16:3ω4, and EPA (Figure 2.6B), while eggs from the TS treatment were lower in 18:1ω9 cis, 18:3ω3, and ARA. Spawned gonads from females in both treatments were higher than feeds in 18:0, ARA, and DHA. Additionally, spawned gonads from the TW treatment were higher in 22:4ω6, and gonads from females fed TS were higher in 20:3ω6, ARA, and 24:1ω9 (Figure 2.6). Spawned gonads from the TS treatment were lower than their feed in 16:0, 18:1ω9 cis, and

18:3ω3 (Figure 2.6A), while spawned gonads from females fed TW were lower than the feed in 14:0, 15:0, 16:0, 16:1ω7, 16:3ω4, and EPA (Figure 2.6B).

2.3.5. Larval Survival and Development

All larvae died within 324 h post-hatching (Figure 2.3). The interaction of feed treatments x time on overall larval survival was not significant ($F = 0.23$, $p = 0.744$). Larvae continued to develop over the course of the experiment regardless of feed treatment; however, larvae from TS-fed females developed slower than larvae from TW-fed females. I observed budding in multiple larval developmental stages occurring at the same time from a single female, in both feed treatments, which could have resulted in extended cohort development with the addition of early larval stages into the larval pool. Approximately 5 % of larvae budded prior to the development of feeding auricularia larvae. There were no morphological differences in larvae between the feed treatments, but budding was slightly more prevalent in the TW feed treatment, which may have resulted in higher survival rates at the transition between pre-feeding (early auricularia) and post-feeding (late auricularia) larval stages (Figure 2.3).

2.4. Discussion

We examined the effects of phytodetritus composition on the reproductive fitness of the holothurian *P. californicus*, including female body condition, egg condition, larval development and survival. Results suggest a trade-off between the number of eggs produced by each female and the fitness of larvae that develop from those eggs. Although female body condition and egg size were similar between feed treatments, females fed the green alga TS had higher fecundity than those fed the diatom TW; however, while larval development rates were similar, TS larvae

had lower survival. Significant differences were also observed in the concentration of the FAs EPA, DHA, 12:0, 16:0, and 18:0 in eggs and spawned gonads of females given different feed treatments. EPA and DHA are important for larval development and could explain the observed differences in larval survival (Hendriks et al. 2003, Ehteshami et al. 2011).

2.4.1. Experimental Simulation of Changing Productivity Regimes

Phytoplankton community composition is influenced by oceanographic conditions, including nutrient distributions as well as temperatures and salinities (Behrenfeld et al. 2006). In some inshore coastal areas, warming and freshening of surface waters in the Northwest Pacific now favors green algae and flagellates where diatoms were once seasonally dominant (Laws et al. 1988, Morán et al. 2010, Chavez et al. 2011). Galloway and Winder (2015) found that interspecific variation in the quantity and quality of lipids and FAs in live phytoplankton cells was almost 3 times higher than intraspecific variations, suggesting that these shifts in taxonomic composition could have important nutritional consequences for consumers including allocation of resources to reproduction and growth.

The two different commercially produced algal monocultures I used as experimental feeds differed in lipid content and FA composition, which can have nutritional consequences for reproductive processes in marine invertebrates. For example, levels of PUFAs, including DHA, in marine invertebrate diets are important to the development and growth of larvae and to the development of reproductive organs (Prowse et al. 2009, Parrish 2013). The diatom feed TW was higher in total lipid than the green alga TS, but TS was higher in the essential FAs ARA and EPA, and total PUFA, while TW was higher in DHA and SFA. These differences found in the feed treatments were reflected in the spawned eggs and gonads of females in each experimental

treatment. Total lipid values for experimental feeds were within ranges previously published for the same species (Reitan et al. 1994, Lee et al. 2010, Wahlen et al. 2011). Interestingly, I found that the TS feed used in this study did not contain detectable levels of DHA, which is typically higher in green algae and flagellates than in other algal taxa (Brown et al. 1997, Guschina and Harwood 2009).

One of a few *in situ* studies that directly tested the effects of shifting phytoplankton communities on reproductive potential occurred in the Baltic Sea (Ahlgren et al. 2005). In this mesocosm study, regional eutrophication caused a shift in the phytoplankton community composition from diatoms to flagellates, resulting in fluctuations in the relative abundances of FA throughout the food web, and possibly leading to a decrease in reproductive potential of Atlantic salmon stocks (Ahlgren et al. 2005). Although the monocultures used in my feed treatments do not fully represent natural *in situ* feeding conditions of a mixed detrital pool, the differences between treatments illustrate that shifts in dominant algal taxa may affect reproductive conditions in marine invertebrate populations as well.

Sediment lipid concentrations in experimental tanks were higher than average values reported globally from natural sediments (0.08 - 1.60 mg lipid g^{-1} sediment dry weight; e.g., Pusceddu et al. 2009), suggesting that animals were not food-limited during the course of the experiment, and observed treatment effects were most likely due to differences in food quality and not food quantity. Sediment lipid and FA content in my experimental tanks may also have been modified by bacterial communities that developed over the course of the experiment, as indicated by the presence of bacterial FA (i.e., odd chain FA, 14:1ω7, and 14:1ω9) in egg and gonad tissues (Burdige 2007, Lipp and Hinrichs 2009, Huettel et al. 2013). *In situ*, phytodetritus also becomes modified by bacterial communities (Russell and Nichols 1999), and bacteria FA

can be incorporated into *P. californicus* tissues (Chapter 3). Here, the presence of bacterial FA in reproductive tissues (eggs and spawned gonads) suggests that bacteria may be an important food source for *P. californicus*, and odd chained bacterial FA may need to be incorporated into commercial feeds.

2.4.2. Effects of Diet on Female Body Condition

Female body condition did not significantly differ between feed treatments, suggesting that both feeds kept females in the same general health. Females in both treatments did lose body weight over the course of the experiments, potentially due to egg production or stress. Nonetheless, weights of spawned experimental females were not significantly different from those of wild females collected from the same site during the normal spawning period (July 2013). However, despite similar body size, total lipid content of muscle and skin was two times lower in experimental females than in wild-caught females (Chapter 3). The lower lipid levels in body wall in both feed treatments may indicate poorer overall female condition compared to wild females. Holothurians do not possess energy-storage organs like some other echinoderms (e.g., pyloric caeca in sea stars; Giese 1959), but it is widely assumed that holothurians store nutrients, including lipids, in body wall tissue for use in growth and reproduction (Ginger et al. 2000, Drazen et al. 2008). Experimental females may thus have mobilized stored lipids from body wall for reproduction, possibly as a result of insufficient FA supplies or the absence of a specific nutrient in the supplied diet.

P. californicus egg development occurs over the course of three years in a "conveyer" like development in gonad tubules (Smiley and Cloney 1985, Smiley 1988). Year one and two are the initial formation of egg cell walls (i.e., phospholipids [PL] use) in gonad tubules, while

year three is the development of egg energy reserves (i.e., triglycerides [TAG]) for initial larval development during non-feeding periods. Egg and gonad FA composition data suggest that when provided a diet of green algae (TS), *P. californicus* may be incorporating higher levels of PUFAs into year one and two egg development (i.e., unspawned gonad tissue) than into year three egg energy reserves (i.e., spawned eggs).

The higher abundance of some FAs in spawned gonads that were low or not detected in feeds suggests that these FAs may have been biosynthesized by *P. californicus* (Allen 1968, Monroig et al. 2013), or transferred to reproductive tissues from other body tissues over the period of the 32-week feeding experiment. Spawned gonads from females in both treatments had higher concentrations of in 18:0, ARA, and DHA compared to feeds. Furthermore, spawned gonads from the TW treatment were higher in 22:4ω6, and spawned gonads from females fed TS were higher in 20:3ω6, ARA, and 24:1ω9 (Figure 2.6). SFAs including 18:0 can be formed *de-novo* in tissues of marine invertebrates (Kelly and Scheibling 2012, Monroig et al. 2013). PUFAs that originate from primary producers can be modified through trophic upgrading (e.g., elongation, saturation, selective uptake) as they transfer through the food chain (Budge et al. 2006, Kelly and Scheibling 2012). For example, Ginger et al. (2000) found that the PUFAs EPA and ARA were absent from sediment, but present in holothurian tissues, suggesting that these compounds could have been acquired during "pulses" of fresh algae detritus or biosynthesized *de-novo*. In addition, marine bacteria isolated from intestines of cold-water invertebrates can produce PUFAs, making it difficult to determine if the source of dietary PUFAs is through *de-novo* synthesis or their bacterial symbionts releasing FA into intestinal tracks of hosts (Das 2006, Monroig et al. 2013).

PUFAs (e.g., EPA and ARA) dominate gonad FA composition of shallow-water holothurians collected from temperate and tropical waters (Ginger et al. 2000, Drazen et al. 2008). Spawned gonads from both treatments were enriched in FAs 20:3ω6, ARA, EPA, 22:4ω6, and DHA relative to experimental feeds. Additionally, spawned gonads from TS fed females had higher overall concentrations of DHA, ARA, ΣSFA, and ΣPUFA, compared to the spawned gonads of the other treatment. This may be an indicator that females fed TS maintained more FA reserves, compared to females fed TW who allocated higher overall levels of these FA to their spawned eggs instead of reserving FA within their gonads.

2.4.3. Maternal Diet Effects on Egg and Larval Condition

Female diet affected the number of eggs produced per female, but not the size of eggs. In contrast, studies on other species of marine invertebrates found that females fed diets high in ω3 FA produced larger eggs with a greater organic matter (OM) and total lipid content (George 1994, de Jong-Westman et al. 1995). In my study, egg FA concentrations were highly variable between samples from the same experimental feed treatment, which has also been observed in other studies and attributed to maternal stress (Poorbagher et al. 2010). Maternal nutritional stress lowers organic content of eggs, often altering egg size and abundance, as well as slowing the initial growth of larval crustaceans, echinoderms, and mollusks (Moran and McAlister 2009). For example, red abalone (*Haliotis rufescens*) females fed a higher lipid diet produced eggs with higher dry weight, lipid and protein, as well as increased hatching success, compared to eggs produced by mothers fed a diet that contained lower lipid content, but also did not have a significant difference in egg sizes (Buchal et al. 1998, Poorbagher et al. 2010). While it is

unclear if maternal stress caused the differences observed in my experimental animals, it cannot be ruled out.

For planktotrophic larvae, including *P. californicus* larvae, feeding stages are only reached after the differentiation of the ciliary feeding apparatus, digestive tract, and enzyme systems (Strathmann 1971). This differentiation requires energy, typically stored in eggs as TAG (Hendriks et al. 2003). Maternal nutrition, and subsequently investment into eggs, can affect the duration of larval periods by affecting growth and development rates, as well as in some cases, larval formation.

Larval survival was similar between the feed treatments, until larvae reached feeding stages and entered into starvation periods, where larvae from TW-fed females had higher survival rates then larvae from TS at a given time point. I also observed *P. californicus* larvae budding, but at lower rates (5 % of larvae) compared to 12 % of laboratory-reared larvae as reported by Eaves and Palmer (2003). Total lipid was four times higher in eggs from females fed TS than in eggs from females fed TW treatment, although not significantly different due to high variance among individual samples. FAs contributing most to dissimilarities in egg FA composition between feed treatments included the SFAs 12:0, 14:0, 16:0, and 18:0, which were higher in eggs from the TW treatment than the TS treatment. ΣSFA concentrations have been linked to increased larval growth and survival because the short carbon chains of some SFA are readily used as an energy source for development (Wehrtmann and Graeve 1998, Ehteshami et al. 2011). The higher concentrations of SFA could explain why larvae from eggs produced from females fed TW had higher survival than those from females fed the TS feed.

Eggs from TW fed females also had higher levels of ARA, EPA, DHA, and PUFA than eggs from females fed TS. The higher levels of PUFA in TW eggs could explain why the

resulting larvae had higher survival, and slightly increased larval development rates, although not significantly different. Previous studies found a connection between increased levels of PUFA in eggs and higher larval survival from these eggs (Ehteshami et al. 2011). ARA, similar to EPA, is needed for the formation and function of eicosanoids (hormone-like compounds needed for growth and development; Bell and Sargent 2003). In both feed treatments, eggs tended to preferentially incorporate essential FAs DHA, EPA, and ARA from feed treatments, so that the levels of these FA were higher in eggs then the corresponding feed treatments. Other studies have also observed the preferential incorporation of DHA, ARA, and EPA (Hendriks et al. 2003, Ehteshami et al. 2011), which can influence gene expression, regulating the synthesis of eicosanoids and other hormones, which could directly affect larval development from eggs, gonad development, and spawning synchronization (Parrish 2013).

2.5. Conclusions

Changes in the species composition of phytoplankton assemblages may have important nutritional consequences for marine consumers. As documented in this study, *P. californicus* maternal investment in females fed the green alga TS produced more eggs of the same size and larvae that had slightly lower survival but similar development rates compared with females fed the diatom TW. Such reproductive plasticity can increase population fitness when there are adequate environmental cues (i.e., food quantity) that can trigger changes in reproductive strategies. Examples of oceanographic conditions that could result in these shifts include regional warming and freshening of surface waters, which favors small phytoplankton taxa such as green algae and flagellates over diatoms (Laws et al. 1988, Morán et al. 2010, Chavez et al. 2011). If environmental conditions are favorable to larvae, then producing a larger number of

less energy-dense eggs (such as in the TS feed treatment) would be beneficial to the population, because planktotrophic larvae could feed on phytoplankton. In contrast, if larval food is less abundant, producing a smaller number of more energy-dense eggs (such as in the TW feed treatment) would be favored, because larvae would be more dependent on egg resources for energy than on the environment.

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2.7. Figures

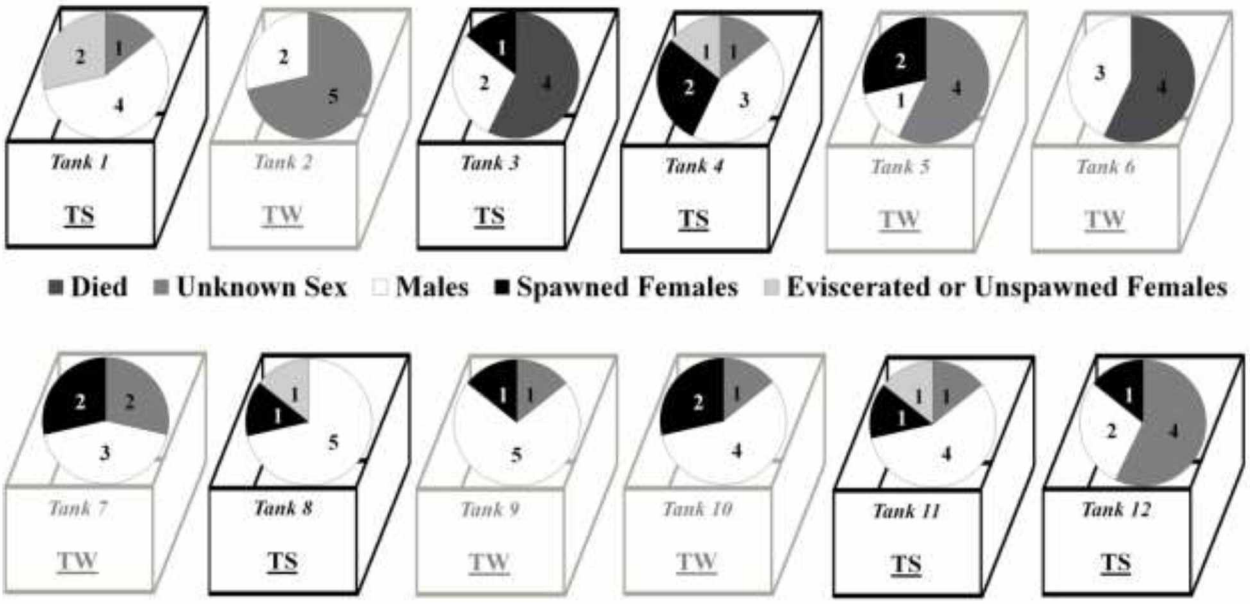

Figure 2.1. Experimental design. Animals in twelve tanks were fed either *Tetraselmis* sp. (TS) or *Thalassiosira* sp. (TW), as indicated. Pie charts denote the composition of animals within tanks over the course of the study.

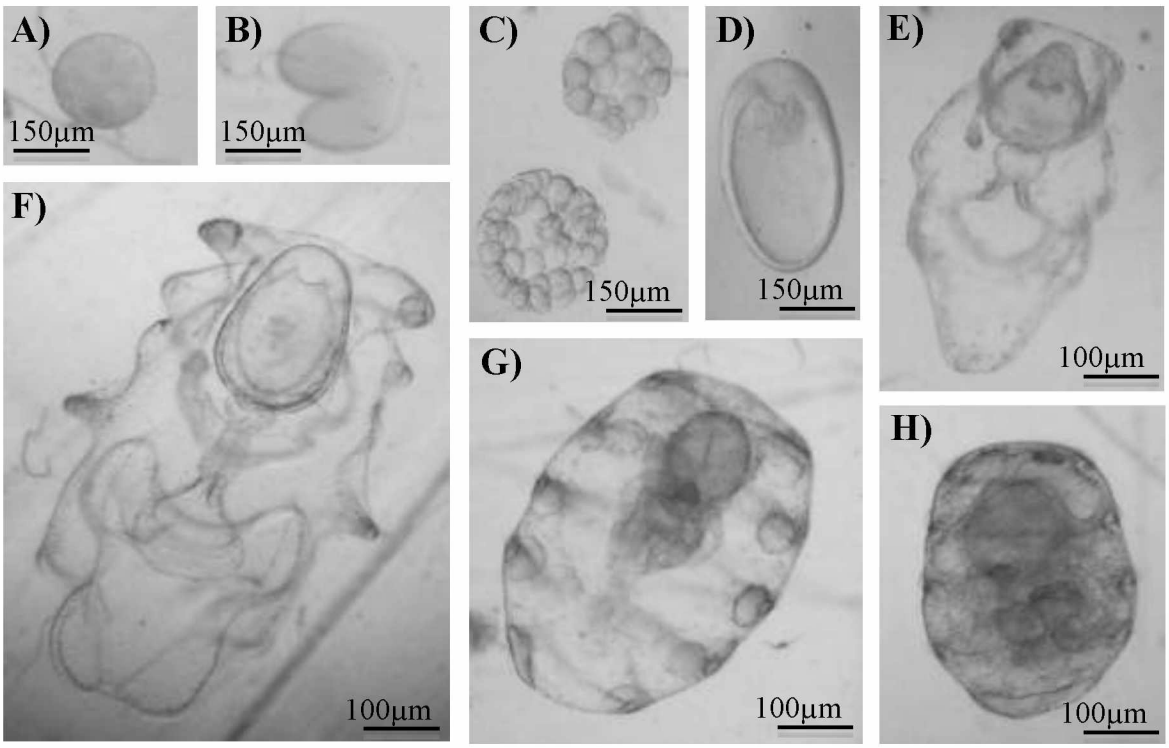

Figure 2.2. Larval development. A) Fertilized egg, B) First cleavage, C) 36-cell embryo, D) early gastrula, E) Early auricularia, F) Late auricularia, G) Doliolaria, H) Late doliolaria.

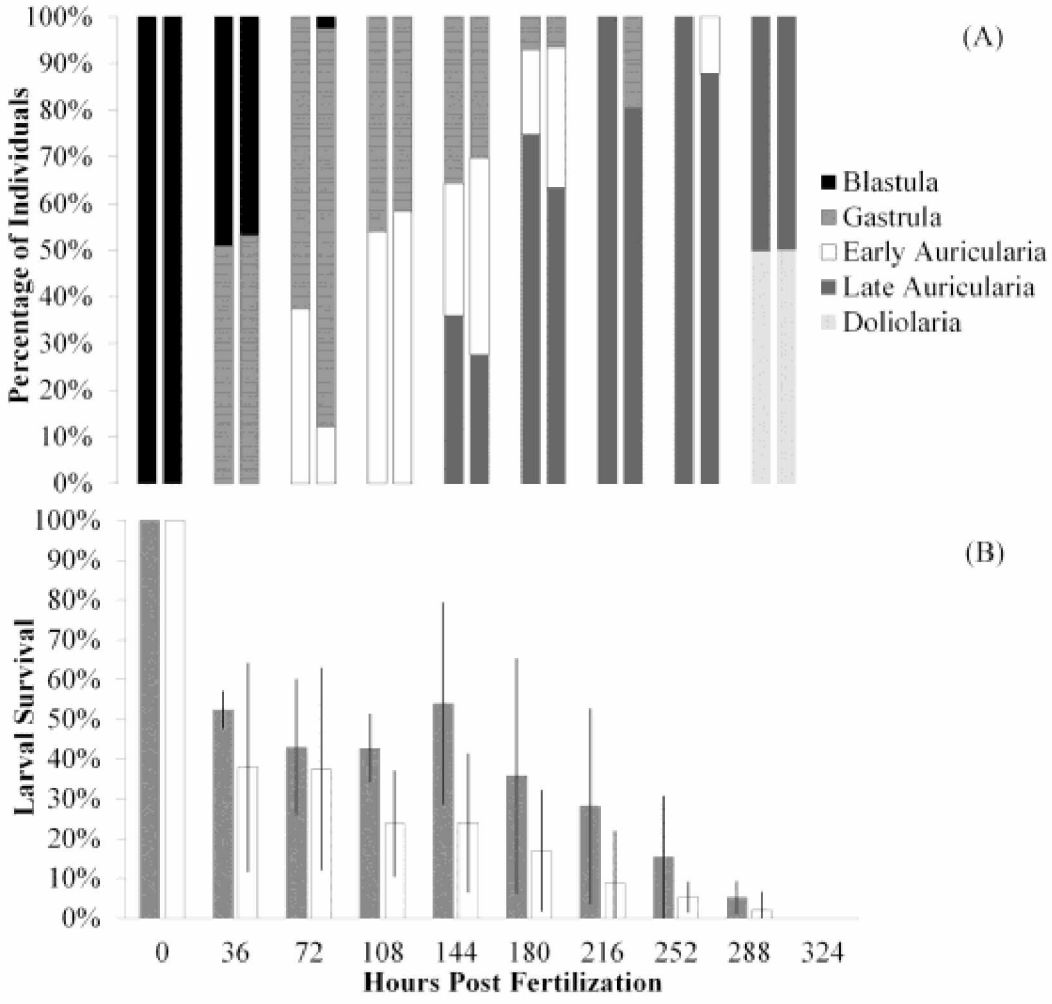

Figure 2.3. Mean percent pre-feeding larval survival, and relative percentage of larvae at each developmental stage . (A) Relative percentage of larvae at each developmental stage, and (B) Mean percent pre-feeding larval survival ± standard deviation (shaded bar *Thalassiosira* sp. [TW]; white bar *Tetraselmis* sp. [TS]) for 0 - 324 h post fertilization.

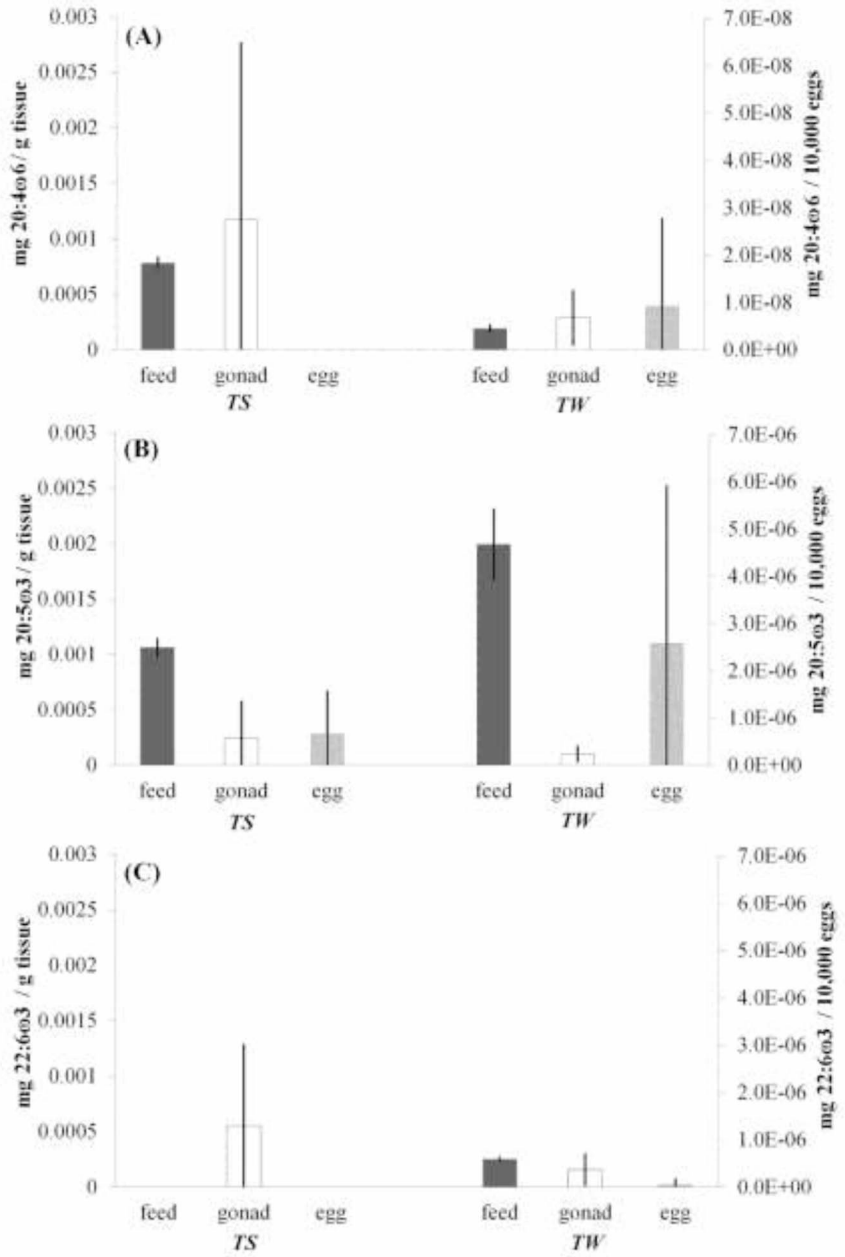

Figure 2.4. Mean FA concentrations in feeds, and spawned gonads and eggs (ARA, EPA, DHA). Mean FA concentrations ± standard deviation in feeds (TS or TW) and *P. californicus* spawned gonads and eggs after being fed TS or TW. Feeds and spawned gonads were measured in mg FA / g tissue (left axis) and eggs were measured in mg FA / 10,000 eggs (right axis). Algal feeds (n = 3 per feed type); Spawned gonads (TW [n = 5]; TS [n = 4]); Eggs (TW [n = 5]; TS [n = 4]). (A) ARA, (B) EPA, and (C) DHA.

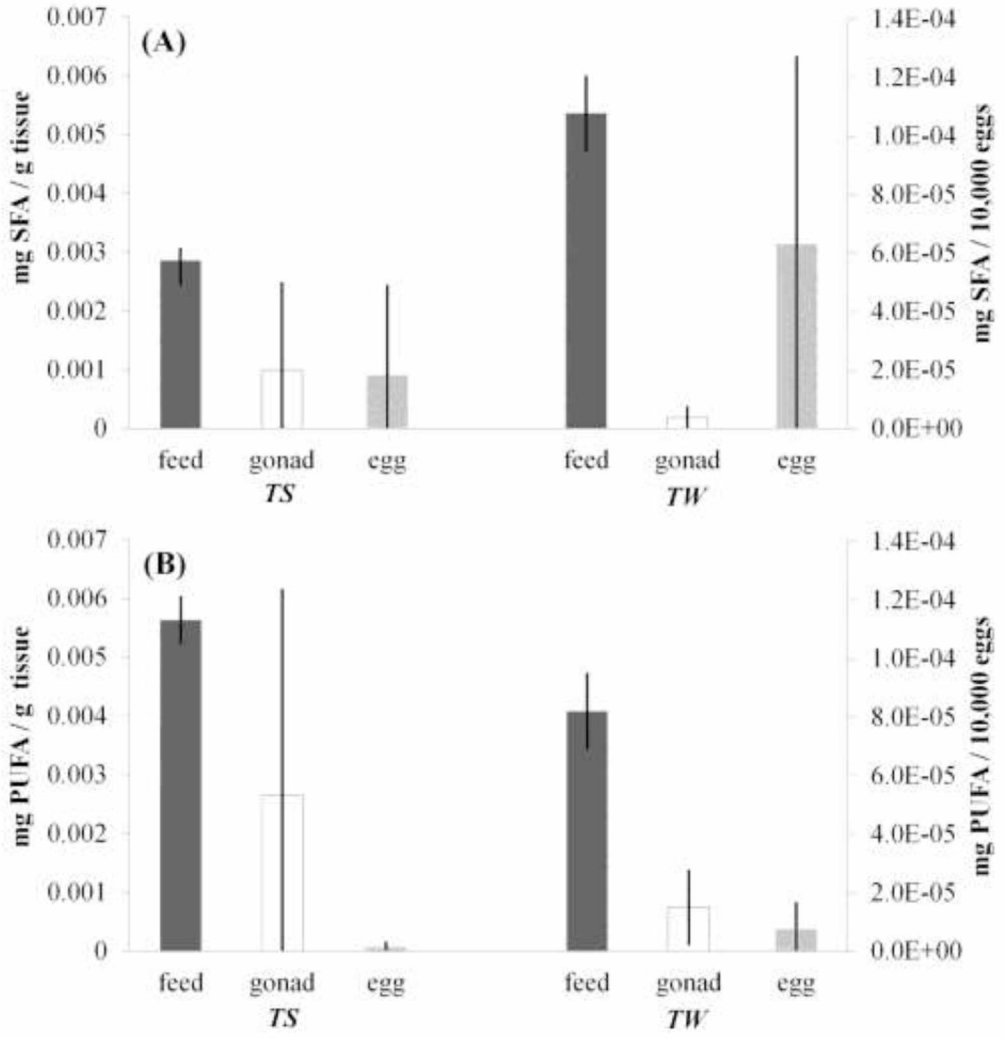

Figure 2.5. Mean FA concentrations in feeds, and spawned gonads and eggs (Σ SFA, Σ PUFA). Mean FA concentrations ± standard deviation in feeds (TS or TW) and *P. californicus* spawned gonads and eggs after being fed TS or TW. Feeds and spawned gonads were measured in mg FA / g tissue (left axis) and eggs were measured in mg FA / 10,000 eggs (right axis). Algal feeds (n = 3 per feed type); Spawned gonads (TW [n = 5]; TS [n = 4]); Eggs (TW [n = 5]; TS [n = 4]). (A) Σ SFA and (B) Σ PUFA.

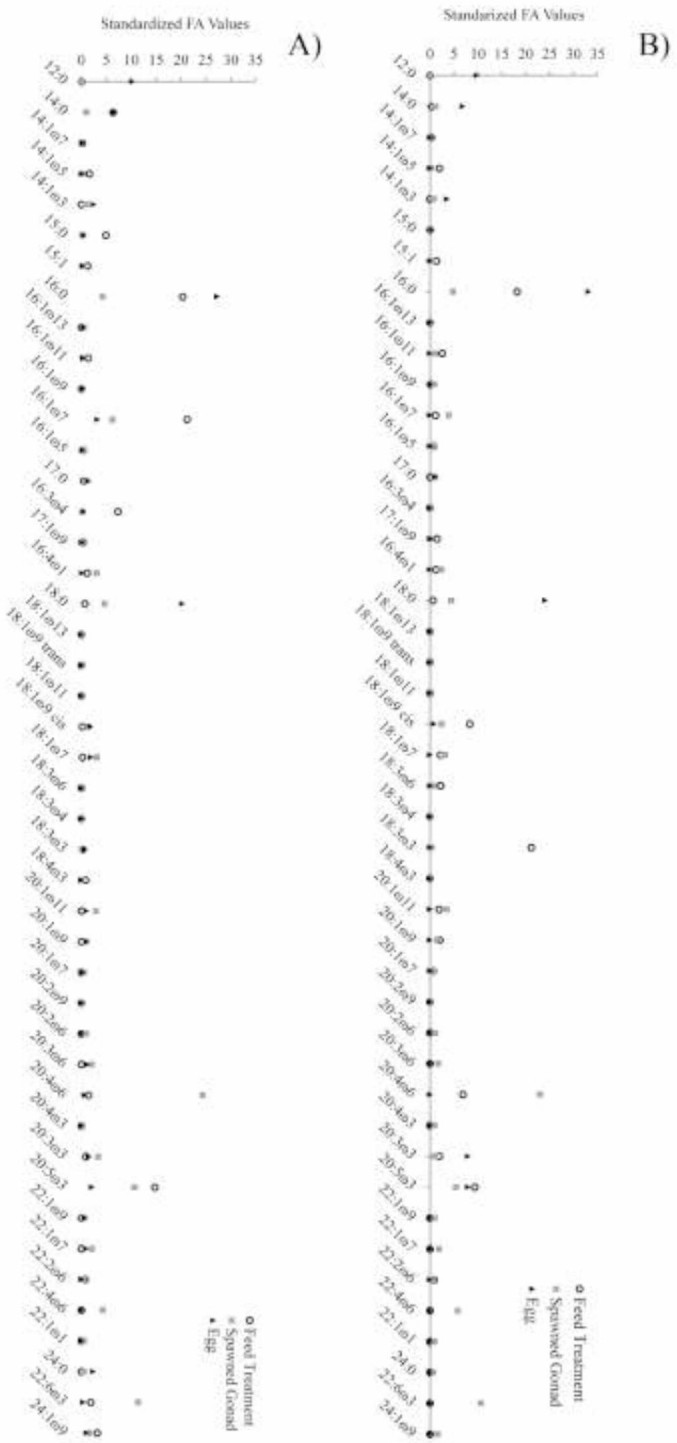

Figure 2.6. Mean values of standardized FA data. Data is for algal feed treatments (white circles), spawned gonads (gray squares), and eggs (black triangles); (A) TS feed treatment; (B) TW feed treatment. See Appendix B for actual values.

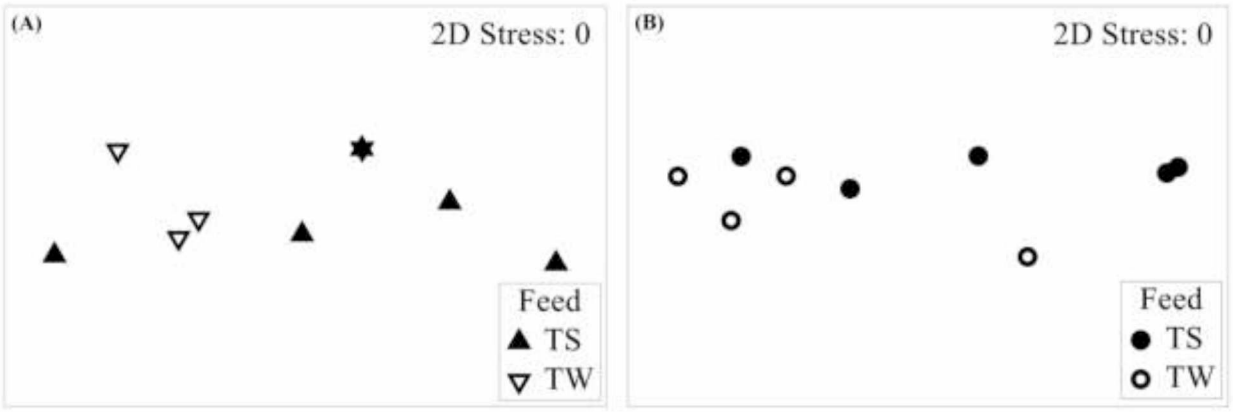

Figure 2.7. Non-metric multidimensional scaling plots. (A) spawned gonads and (B) eggs by algal feed treatments (TW [n = 5]; TS [n = 4]). Each point reflects the FA signature (including all 67 identified FA) for each replicate with some replicates overlapping.

2.8. Tables

Table 2.1. Mean values for biochemical measurements of algal feed treatments. Data shown ± standard deviation (n = 3 per feed). Organic matter (OM), Carbon (C), and Protein (P).

Phytoplankton Feed	***Tetraselmis* sp.**	***Thalassiosira* sp.**
dry wt. (g) / concentrate (ml)	0.11 ± 0.01	0.05 ± 0.01
OM (%)	69.10 ± 0.11	48.42 ± 0.16
C (%)	32.71 ± 0.84	23.24 ± 0.10
P (%)	32.58 ± 0.03	18.79 ± 0.12
mg lipid / g dry wt.	4.60 ± 0.82	7.15 ± 1.81
mg total FA / g dry wt.	4.43 ± 0.64	3.92 ± 0.33

Table 2.2. Female body and egg condition variables by female based upon algal feed treatments. Mean values ± standard deviation. Separate Student's t-tests were performed between TW (n = 5) and TS (n = 4). * denotes significant comparison.

Condition Variable	*Tetraselmis* sp.	*Thalassiosira* sp.	p-value
post-spawn gonad index (GI)	2.74 ± 2.54	2.25 ± 1.87	0.905
total tissue g wet wt.	98.96 ±19.99	104.85 ± 21.51	0.904
total tissue moisture (%)	89.20 ± 1.14	88.71 ± 0.31	0.191
mg lipid / g gonad wet wt.	6.22 ± 5.56	5.58 ± 1.82	0.678
mg total FA / g gonad wet wt.	4.39 ± 1.72	4.88 ± 1.68	0.999
mg lipid / 10,000 eggs	0.04 ± 0.04	0.01 ± 0.01	0.111
mg lipid / g muscle wet wt.	16.65 ± 5.27	17.69 ± 6.35	0.730
mg lipid / g skin wet wt.	7.38 ± 2.69	8.21 ± 1.56	0.556
eggs / g female wet wt.	2,308.43 ± 699.75	1,121.38 ± 411.29	**0.016***
egg diameter (µm)	160.02 ± 17.83	165.58 ± 14.04	0.190
egg fertilization (%)	93.60 ± 5.29	89.10 ± 18.43	0.262

Table 2.3. SIMPER analysis of percent dissimilarities in FA composition between algal feeds, and between spawned gonads and eggs from each experimental feed treatment. Separate SIMPER analysis was conducted per sample type. Only FAs contributing more than 4.5 % of total dissimilarity were noted below.

Sample	FA	Contribution to Total Dissimilarity (%)
Algal Feed Treatments (57.55 % mean dissimilarity)	16:1ω7	19.05
	18:3ω3	16.60
	16:3ω4	6.91
	18:1ω9 cis	6.42
	EPA - 20:5ω3	6.40
	14:0	5.67
Spawned Gonads (73.16 % mean dissimilarity)	ARA - 20:4ω6	22.37
	DHA - 22:6ω3	10.70
	EPA - 20:5ω3	6.03
	22:4ω6	5.44
	16:0	4.81
	16:1ω7	4.67
Eggs (79.70 % mean dissimilarity)	16:0	28.20
	18:0	20.47
	12:0	9.03
	14:0	6.40

Chapter 3. Dietary Lipid Uptake and Utilization in the Commercially-Harvested California Sea Cucumber *Parastichopus californicus* (Stimpson 1857) in Southeast Alaska

Abstract

The exact causes for population declines of commercially-harvested *Parastichopus californicus* (the California sea cucumber) in the Gulf of Alaska are unknown. While many factors have been proposed (e.g., fishing pressure and sea otter predation), changes in the quantity and/or variety of food may also be a contributing factor. Nutritional stress may result from seasonal or climate-driven changes in primary production, and direct effects of such changes on reproductive potential and overall health have been documented in several invertebrate taxa. I investigated the effects of diet on reproduction in *P. californicus*, which undergoes a natural cycle of viscera reabsorption and cessation of feeding during winter months when females likely rely on stored lipids and fatty acids (FA) for initial gonad development. Adult females were collected from Southeast Alaska (SEAK) at eight time points from April 2012 – July 2013, encompassing a full reproductive cycle. Gut content mass and composition were used to assess temporal patterns in diet. Patterns in lipid storage and utilization were determined through total lipid and specific FA analysis of gut contents, gonad, muscle, viscera, and skin tissues. I tested three hypotheses: 1) bulk gut contents and ratios of gonad, viscera and body wall weight to total animal weight vary with reproductive period, 2) total lipid content of body tissues and bulk gut content vary seasonally gonad lipid content, 3) temporal patters in FA composition of body tissues mirror patterns in gonad maturation period. I found that all tissue ratios varied significantly among collection dates and gonad maturation periods. Shell debris,

fine particulate matter (≤ 1 mm), and terrestrial debris were the most abundant contents found in all guts regardless of gonad maturation period or collection date. FA composition and concentrations of specific key FA differed significantly among gonad maturation periods and collection dates in skin, viscera, and gonads, suggesting that both skin and viscera may store lipids used for gonad development. Lipid storage in viscera may explain the seasonal absorption of visceral mass observed in this species. These results demonstrate the relevance of dietary factors for reproductive fitness in coastal benthic deposit feeders.

3.1. Introduction

In the Gulf of Alaska, commercially-harvested populations of *Parastichopus californicus* (the California sea cucumber) are in decline, exhibiting decreases in both body weight and abundance (Clark et al. 2009, Hebert 2014, O'Regan 2015). While the exact causes for population changes have not been identified, predation by sea otters (Larson et al. 2013) and fishing pressure (Anderson et al. 2011, Friedman et al. 2011) have been suggested. Dietary shifts (i.e., changes in the quantity and/or variety of food) may also be a contributing factor, resulting in nutritional stress that can reduce growth, survival, and reproductive output. Food quality affects female reproductive potential, and to some extent growth, in several marine invertebrates (Hendriks et al. 2003, Goedkoop et al. 2007, Lester et al. 2007).

Variations in oceanographic conditions in the Gulf of Alaska force shifts in pelagic primary production, for example due to the Pacific Decadal Oscillation (PDO; Stabeno et al. 2004, Sturdevant et al. 2012). During "warm" phases of the PDO, positive seawater temperature anomalies along the coast cause a deepening of the mixed layer, and increased overall primary production, especially of diatoms; the opposite is generally true for a "cold" phase PDO (Stabeno

et al. 2004). Such shifts in regional diatom biomass have been linked to fluctuations in population size and reproductive potential of other Alaskan fishery species, including several species of large crab (Zheng and Kruse 2006, Moloney et al. 2011) and salmon (Hare et al. 1999). Diatoms and diatom detritus are a primary food source for benthic deposit feeders in subtidal soft-sediment habitats, and a substantial contributor to sediment lipid pools (Shimanaga et al. 2000, Lipp and Hinrichs 2009). Anthropogenic changes, including shoreline deforestation in the coastal areas surrounding the Gulf of Alaska may also be affecting marine invertebrate food supplies (Schoonmaker 1997, Albert and Schoen 2013). Terrestrial matter can be an important source of carbon and lipids into marine ecosystems (Baldock et al. 2004, Blair and Aller 2012). However, logging activity has increased in Alaska in the last several decades, which could decrease terrestrial matter input over time (Albert and Schoen 2013).

Nutritional needs are poorly quantified for most deposit feeders, including *P. californicus*, which depends heavily on phytodetritus flux to the benthos and detritus-associated micro-organisms (Yingst 1982). However, composition of the diet is difficult to determine in deposit feeders that consume varied and largely unrecognizable food particles. A combination of gut content analysis and biomarker techniques can be informative for diet studies in deposit-feeding species (Hyslop 1980, Budge et al. 2002).

Historical data from British Columbia, Canada, indicate that *P. californicus* ceases feeding in winter and undergoes an annual re-absorption of the viscera. The timing of these events appears to coincide with gonad maturation prior to the early summer spawning season (Fankboner and Cameron 1985, Cameron and Fankboner 1986), suggesting lipids stored in reabsorbed tissues may be used to fuel this energetically demanding process. Mobilization of stored lipids may release *P. californicus* from dependence on seasonally variable food resources

that are less abundant in late fall and winter (Strom et al. 2015), and allow it to enter a period of dormancy during low-food months (Cáceres 1997).

In several sea cucumbers and other echinoderm species, lipid and fatty acid (FA) stores build up in the body tissues during feeding months and support gonad ripening during non-feeding months prior to the annual spawn (Neto et al. 2006, Smith 2008, Xu et al. 2016). Energy storage is often associated with an increase in total lipid, as well as an increase in the physical mass of tissues (i.e., tissues ratios [the proportion of a specific tissue to the total body weight]). Sea cucumbers have no dedicated energy storage organs (e.g., like the pyloric caeca in sea stars), and must store nutrients in other body tissues (Lawrence 1976, Oudejans and Van der Sluis 1979). In sea urchins, gonads store lipid for reproduction (Hughes et al. 2006), but in *P. californicus* absorption of visceral tissue could also mobilize stored energy and nutrients from both the tissues and the microorganisms that line the intestinal tract (seen in fish; Yano et al. 1994). *Cucumaria frondosa*, a commercially harvested sea cucumber in the North Atlantic, has been found to undergo winter cessation of feeding and mobilize lipids and proteins from structural body tissues to support gonad development (David and MacDonald 2002).

Patterns in lipid storage and utilization are particularly informative in examining environmental effects on reproductive output. Lipid content of both diet and body tissues have been directly linked to total reproductive output of several populations of commercially harvested invertebrates (e.g., shrimp, scallops, and sea cucumbers; Whyte et al. 1990, Xu et al. 1994, Ying et al. 1998, David and MacDonald 2002, Xu et al. 2016). FA content of the diet also influences fecundity, egg size, and FA composition of spawned eggs (Wehrtmann and Graeve 1998, Hendriks et al. 2003, Ehteshami et al. 2011). In particular, many FA essential for growth and reproduction cannot be readily synthesized by animals (Budge et al. 2006). These essential

FA (e.g., 22:6ω3 docosahexaenoic acid (DHA) and 20:5ω3 eicosapentaenoic acid (EPA)) must, therefore, be acquired from the diet (Parrish 2013). These and other omega-3 FAs influence gene expression regulating the synthesis of eicosanoids and other hormones, which could directly affect larval development and spawning synchrony (Parrish 2013).

The objectives of this study were to examine trends in feeding (both ingestion and composition of ingested food items) of *P. californicus* populations in Southeast Alaska (SEAK), and consider whether body tissues store nutrients used to fuel annual reproductive events. In particular, I looked at FA profiles of body tissues and gut contents to identify key dietary FA that may be partitioned to gonad maturation and egg production. I tested three hypotheses: 1) bulk gut contents and ratios of gonad, viscera and body wall weight to total animal weight vary with reproductive period, 2) total lipid content of body tissues and bulk gut content vary seasonally gonad lipid content, 3) temporal patters in FA composition of body tissues mirror patterns in gonad maturation period.

3.2. Materials and Methods

3.2.1 Collection and Initial Analysis

Adult specimens of *P. californicus* (length > 10 cm; individual wet weight ≥ 120 g) were hand-collected by the Southeast Alaska Regional Dive Fisheries Association commercial divers (SARDFA) in George's Inlet, Southeast Alaska (SEAK; 55° 20' N, 131° 28' W) at depths of 5 - 10 m. Collections for all analyses occurred every two to three months from April 2012 - July 2013, for a total of eight collections encompassing a complete annual reproductive cycle (Cameron and Fankboner 1986). Gonad maturation was defined as spawning, post-spawning, or gonad developing periods (Chapter 1; Figure 3.1). High variance (i.e., 10 – 12 var.) in gonad

ratios in a given month was used as evidence of spawning periods, because it suggests that both reproductively mature (high gonad ratio; i.e., 15 – 25 %) and recently spawned (low gonad ratio; i.e., ≤ 1 %) individuals were present in the same population. Small gonad ratios with low variance (i.e., ≤ 2 var.) denoted post-spawned periods, while medium to large gonad ratios (i.e., 5 – 10 %) with some variance (i.e., 4 – 8 var.) denoted developing periods.

Live animals were placed in groups of five into plastic bags filled with seawater (7 - 8 °C), and transported in coolers via air cargo to the University of Alaska Fairbanks, Seward Marine Center. Animals arrived in Seward, AK within 15 h of collection. Some animals eviscerated (i.e., internal organs expelled through the mouth, including respiratory tree, intestinal gut, and rete mirabile) during transport and were excluded from my study. Although evisceration does not kill *P. californicus* (Fankboner and Cameron 1985), I wanted to obtain intact viscera tissues for analysis. Females were targeted for this study, yet sex cannot be determined from external morphology. In this case for each of the collections, animals were sacrificed at random until 5 - 10 females were obtained. Animals were dissected and gonad, skin (dermis and connective tissue), muscle, and viscera were separated and weighed wet using a Mettler-Toledo balance (model PB3002-S, accuracy to 0.001g). Tissue ratios were calculated as the ratio of tissue wet weight to whole body wet weight.

3.2.2. Gut Content Analysis

Gut contents were separated from gut tissue and contents were weighed and identified. The presence or absence of diatoms was noted based on visual inspection of wet gut contents using a glass slide smear of samples and a Leica DM2000 compound microscope. Diatoms were incorporated into analyses of total gut content weights but not the percentages of prey types since

it was not possible to get an accurate weight of diatoms. Gut contents were then freeze-dried using a VirTis Freeze Dryer (model 52; The VirTis Company) for 32 h. Freeze-dried gut contents were weighed on a Mettler-Toledo balance (model PB3002-S) for total dry mass, and then contents were passed through a 1-mm mesh sieve. Gut contents passing through the sieve were reweighed and defined as gut content ≤ 1mm. Gut contents larger than 1mm were identified to the lowest possible taxonomic level, and each prey type was weighed separately. Total gut content dry weight for each individual was divided by the total viscera wet weight to normalize data from females of different body size. Percent composition of each prey type was recorded relative to total gut content dry weight. Gut contents were classified into three categories: gut content < 1 mm, terrestrial debris (wood matter and pine needles), and shell debris.

3.2.3 Total Lipid and FA Analysis

Body tissues (skin, muscle, gonad, and viscera) and bulk gut contents were analyzed for total lipid concentration as well as concentration of individual FAs (mg g^{-1} dry tissue) to determine whether seasonal patterns corresponded to an annual gonad maturation cycle. Total lipid was defined as the weight of the bulk lipid complexes (e.g., lipoproteins, phospholipids [PL], triglycerides [TAG], and wax esters) that were extracted from samples during lipid extraction procedures. All samples were frozen at -40 °C until processing, and then freeze-dried in a VirTis Freeze Dryer (model 52; The VirTis Company) for 32 h. A MettlerToledo analytical balance (model PB3002-S) was used for all biochemical mass determinations. Lipid extractions were performed using a Dionex™ Accelerated Solvent Extractor (ASE 200) operated at two 5-min static cycles, 85 °C furnace, and under 1500 psi N_2 gas according to methods by Dodds et al.

(2004). Samples were mixed with equal amounts of Chem-tube hydromatrix drying agent (Varian INC) prior to lipid extraction. Skin and muscle samples were extracted using 33 mL ASE cells (1.0 g dry weight per replicate), while gonad, viscera, and gut content were extracted using 11-mL cells (0.5 g dry weight per replicate). Dichloromethane (DCM) was used as the extraction solvent (99.98 % purity Fisher Thermo Scientific™), which was amended with butylated hydroxytoluene (Sigma Chemical) at a concentration of 100 mg L^{-1} in DCM to prevent lipid oxidation. Total lipids were concentrated in a TurboVap® LV solvent evaporator (Zymark INC) under N_2 gas operated at 36 °C for 2 h, and then weighed to gravimetrically determine weight of total lipid from dry tissue weight.

FAs were converted to FA methyl esters (FAME) using an acid-catalyzed esterification with Hilditch reagent according to Iverson et al. (2002), and quantified using gas chromatography (GC) coupled to a flame ionization detector (FID) with identical chromatographic conditions as described by Farrugia et al. (2015). A maximum of 20.0 mg lipid was esterified from each sample replicate. Briefly, FAMEs were concentrated using a TurboVap® LV solvent evaporator (Zymark INC) and FAME weights were recorded prior to identification by injection into an Agilent model 6850N Series II GC-FID fitted with a DB-23 (60 m × 0.25 mm, 0.25 µm film) capillary column (Agilent Technologies). Peak areas of 37 FAs were identified by comparing retention times to those of the external standard (Supelco 189-19) FAME mixture. Response factors were calculated for FAs and used to correct peak areas of FAMEs detected in samples following the method described by Ackman and Sipos (1964). The resulting equations for the calibration curves are shown in Table A-1. Remaining FAs were identified using a GC model 6890 interfaced with a mass spectrometer (MS) detector model 5973N (Agilent Technologies) and comparing mass spectra of FAMEs to spectra in the MS

Library (05 v.2.0). A total of 67 FAs, comprising ≥ 95% of the total peak areas of the GC-FID spectral run, were quantified. Results were compiled as FA concentrations using linear calibration curves determined for FAs in an external standard mixture (Supelco 189-19). For FAs identified in samples by GC-MS that were not present in the external standard mixture, calibration curves for FAs of same carbon chain lengths and number of double bonds were used (e.g., 20:1ω9 linear equation was used to quantify 20:1ω7). If no similar FA was present, then the given FA was removed from further analysis. FA composition were only analyzed for tissue types (i.e., skin, gonad, and viscera) that demonstrated significant differences in total lipid between collection dates or gonad maturation.

3.2.4. Statistical Analysis

A series of statistical tests were conducted to assess patterns in total lipid and FA storage and utilization in different tissue types over the reproductive cycle of female *P. californicus* from SEAK. Individual females were used as replicates for statistical analyses. Diagnostic testing on tissue ratio and total lipid data was performed prior to statistical analyses. If data violated assumptions of normality or linearity, data were transformed accordingly (e.g., x^2 transformed; depending on data set), until normality was reached. FA data were not transformed. Timing of field collections was designed to encompass my best estimates of all phases of the annual reproductive cycle. Collections included gonad development (November and December), spawning (April), and post-spawning (May – October and January - February) periods for 2012 and 2013 and were based on previous published data from British Columbia, Canada (Figure 3.1; Cameron and Fankboner 1986, Cameron and Fankboner 1989). Collection date and gonad

maturation periods were tested separately to determine if observed patterns were due to seasonal cycles or possibly differences between study years (i.e., temporal variations).

Univariate data were analyzed using R software (R Development Core Team 2008). Mean individual wet weights, tissue ratios, and total lipid of each tissue type were compared across collection dates and gonad maturation periods (Figure 3.1) using an analysis of variance (ANOVA) with Tukey post-hoc testing. The significance level for all tests was initially set at $\alpha = 0.05$, but a Bonferroni correction was applied when multiple analyses were conducted on the same sample (i.e., in the case of tissue ratios and total lipid content of each tissue). Mean gut content weight (g gut content g^{-1} viscera wet weight) and total lipid in gut contents were also compared across collection dates and gonad maturation periods using ANOVA with Tukey post hoc testing. Mean concentrations of total saturated FA (ΣSFA), total monounsaturated FA (ΣMUFA), total polyunsaturated FA (ΣPUFA), total terrestrial FA (18:3ω3 and 18:3ω6), total diatom FA (16:4ω1, 16:1ω7, and 16:0), and total bacterial FA (14:1 and those with odd-numbered C chains) were pooled across gonad maturation periods and compared between tissue types (skin, muscle, and gonad) using separate ANOVAs with Tukey post-hoc testing for each FA class.

Pearson Product Moment correlations conducted in R (R Development Core Team 2008) were used to determine how tissue ratios and total lipid contents related to gonad maturation periods. Pearson correlations were conducted between non-reproductive tissue ratios (i.e., muscle, skin and viscera) and gonad ratios from the same time points. Pearson correlations were also conducted between tissues/gut content total lipids and gonad total lipids.

Multivariate FA data were analyzed using PRIMER-E v.6 (Clarke and Gorley 2001). An analysis of similarity (ANOSIM) was used to determine if there were significant differences in

the FA composition of each tissue type (skin, gonad, and viscera). Subsequent similarities percentage (SIMPER) analysis was conducted to determine which FAs contributed most to similarities within a tissue type at a single collection point ($\alpha = 0.05$). Tissue FA composition from skin, gonad, and viscera were then visualized using non-metric multi-dimensional scaling (nMDS), and statistically significant groupings of replicates were identified using a hierarchical cluster analysis with similarity profile analysis (SIMPROF). Only significant groupings are shown on nMDS plots.

3.3. Results

3.3.1. Temporal Patterns in Tissue Ratios and Total Lipid

Mean female wet weights differed significantly among collection dates ($F = 3.529$, $p = 0.004$), but had no clear relationship to gonad maturation period or collection years (2012 or 2013; Figure 3.2). Gonad ratios were significantly different among gonad maturation periods ($F = 16.03$, $p = 0.001$), with gonad ratios in spawning and post-spawning periods significantly different from other times (Figure 3.3A). Viscera ratios were significantly different among collection dates ($F = 25.78$, $p \leq 0.001$), and differences were evident between gonad maturation period (Figure 3.3B). Muscle ratios differed significantly among collection periods ($F = 10.49$, $p \leq 0.001$), but direct relationships to gonad maturation periods were less clear (Figure 3.3C). Skin ratios were also significantly different among collection dates ($F = 10.49$, $p \leq 0.001$), but not gonad maturation periods, predominantly due to the low skin ratios on April-16-2012 (Figure 3.3D). Skin and muscle ratios were strongly negatively correlated with gonad ratios ($\rho = -0.66$, $p \leq 0.001$ and -0.50, $p = 0.002$, respectively), while viscera ratios were strongly positively correlated with gonad ratios ($\rho = 0.54$, $p = \leq 0.001$).

Tissue lipid values did not vary significantly between gonad maturation periods. Total lipid in gonads differed significantly among collection dates ($F = 5.901$, $p \leq 0.001$; Figure 3.4A). The contribution of gonads lipids to total animal lipid varied by gonad maturation periods, from 12 % in spawning seasons to 0.01 % in gonad development periods. Viscera total lipid was also significantly different among collection dates ($F = 16.16$, $p \leq 0.001$), but only due to a large peak on August-26-2012 (post-spawning period; Figure 3.4B). August-26-2012 data were not considered outliers, as the variance at this time point was no larger than at any other time (Figure 3.4A). Muscle total lipid was significantly different among collection dates ($F = 0.96$, $p = 0.047$), but unlike other tissue types, post-hoc testing showed no differences between individual collection dates (Figure 3.4C), and no further FA analysis was conducted on this tissue type. Skin total lipid differed significantly among collections dates ($F = 16.61$, $p = 0.001$; Figure 3.4D). Viscera total lipid was weakly negatively correlated to gonad total lipid ($\rho = -0.33$, $p = 0.059$). Skin and muscle total lipid were also weakly negatively correlated to gonad total lipid ($\rho = -0.37$, $p = 0.024$ and -0.31, $p = 0.023$, respectively).

3.3.2. Temporal Patterns in Gut Content

Females from SEAK continued to feed year-round. The gut content dry weight (g dry gut content g^{-1} viscera wet weight) ranged from 0.10 – 1.25 and significantly differed among collection dates ($F = 4.016$, $p = 0.002$; Figure 3.5A). Total gut content tended to be higher during spawning and post-spawning periods and lower during gonad developing periods (Figure 3.5A). Diatoms were abundant in all gut contents, but could not be separated and accurately weighed as a fraction of the total; therefore, they were included in the gut sediment < 1 mm category for quantitative analysis. The proportion of gut contents attributed to each of the three

categories varied significantly among collection dates (gut sediment < 1 mm: $F = 2.623$, $p = 0.029$; terrestrial debris: $F = 3.33$, $p = 0.009$; shell debris: $F = 3.305$, $p = 0.010$; Figure 3.5B).

Total lipid of the total gut content ranged from 0.001 mg lipid g^{-1} dry gut content on May-31-2013 (post-spawn period) to 0.185 mg lipid g^{-1} dry gut content on July-24-2013 (post-spawn period; Figure 3.5C), and was significantly different among collection dates ($F = 2.547$, $p = 0.033$). Although there were significant post-hoc tests for total lipids in gut content among collection dates, there was no clear relationship between the lipid data and gonad maturation periods (Figure 3.5C); therefore, no further FA analysis was conducted on bulk gut content. Gut content was not correlated with gonad ratio ($\rho = 0.18$, $p = 0.279$) or gonad total lipid ($\rho = 0.08$, $p = 0.622$), suggesting that gonad maturation and lipid storage are not directly linked to feeding activity (measured using the proxy of gut content mass).

3.3.3. Tissue-Specific Differences in FA Composition among Gonad Maturation Periods

Differences in FA composition were examined for each tissue type (gonad, viscera, or skin) among the gonad maturation periods (spawning, post-spawning, and developing). Gonad FA composition differed significantly among collection dates (ANOSIM: $p = 0.002$, Global $R = 0.129$), with lower within-group similarity (i.e., more variation among individuals) during gonad developing periods than in spawning or post-spawning periods (Table 3.1, Figure 3.6A). FA that contributed to at least 50 % of the total within-group similarity among collection dates included: 20:4ω6 (arachidonic acid (ARA)), DHA, 16:0, EPA, and 16:1ω7 (Table 3.1). The concentrations of these FAs tended to be lower when gonad ratios were low (Figure 3.6B, C), with some exceptions, suggesting that gonad index (percent of wet gonad to total body wet weight; GI) and lipid content are not directly correlated.

Viscera FA composition differed significantly among collection dates (ANOSIM: $p = 0.010$, Global $R = 0.584$). Viscera FA composition showed clear separation between spawning periods and post-spawning / developing gonad periods (blue and red symbol coloration, respectively; Figure 3.7A). FAs that contributed at least 50 % of the total within-group similarity among collection dates included ARA, DHA, EPA, and 20:3ω6 (Table 3.2). The relative concentration of ARA and EPA were higher when gonad ratios were higher, coinciding with spawning gonad periods (Figure 3.7B, C).

Skin FA composition differed significantly among collection dates (ANOSIM: $p = 0.010$, Global $R = 0.362$). FA in skin were similar across gonad maturation periods (Figure 3.8A), and showed no pattern relative to gonad ratios (Figure 3.8B). FA that contributed at least 50 % of the within-group similarity for each collection date included 16:0, ARA, EPA, and 22:2ω6 (Table 3.3); however, these FA had no trends with gonad ratio or gonad maturation periods (Figure 3.8B, C).

All FA classes and trophic markers examined were highest in gonad and lowest in viscera tissue, except for Σ PUFA concentrations (Figure 3.9). FA of possible terrestrial origin (18:3ω3 and 18:3ω6) were low but present in all tissues (mean across all sample types 0.163 ± 0.08 mg FA g^{-1} dry sample), showing that female *P. californicus* may incorporate lipids from terrestrial debris into their tissues. Only Σ SFA, Σ diatom FA (16:4ω1, 16:1ω7, and 16:0), and Σ bacterial FA (14:1 and odd C chain FA) differed significantly among tissue types (Table 3.4, Figure 3.9). Skin was similar to both gonad and viscera in Σ SFA and Σ diatom FA, while gonad was similar to both viscera and skin in Σ bacterial FA (Table 3.4, Figure 3.9).

3.3.4. Gonad Maturation Period-Specific Differences in FA Composition among Tissues

FA composition differed significantly among tissue types (gonad, viscera, and skin) during the spawning period (ANOSIM: $p = 0.010$, Global $R = 0.653$). FA that contributed most to the total dissimilarity among tissue types included: 20:3ω6, ARA, 20:4ω3, DHA, and 16:0 (Table 3.5). Viscera clearly differed from skin and gonad tissue (Figure 3.10A), with higher concentrations of ARA and EPA in gonad and skin than in viscera during spawning periods (Figure 3.10B).

During post-spawning and gonad developing periods, FA composition also differed significantly among tissue types (ANOSIM: $p = 0.001$, Global $R = 0.280$; $p = 0.001$, Global $R = 0.384$; respectively). FA that contributed most to total dissimilarity among tissue types in post-spawning periods included ARA, DHA, 16:0, and EPA (Figure 3.11A, Table 3.6). Overall, skin and viscera tissues had lower concentrations of ARA, EPA, and DHA compared with gonad tissue during post-spawning periods (Figure 3.11B). In contrast, FA composition in gonad developing periods clearly differed among gonad, viscera, and skin tissues (Figure 3.12A). FA that contributed most to the total dissimilarity among tissues during gonad developing periods included ARA, DHA, 16:0, and EPA (Table 3.7). The relative concentration of 16:0 separated visceral tissue from gonad and skin tissues during gonad developing periods (Figure 3.12B).

3.3.5. Tissue- and Period-Specific Differences in EPA and ARA Concentration

Temporal changes in ARA and EPA concentrations (mg FA / g dry tissue) were further examined due their importance in distinguishing tissue types and gonad maturation periods. Concentrations of EPA and ARA in skin and gonad did not differ significantly among gonad maturation periods (Figure 3.13A, B; Table 3.8). In contrast, concentrations in viscera

significantly differed across gonad maturation periods (Figure 3.13B, Table 3.8). Viscera tissue during spawning gonad period was depleted in ARA, and then ARA values increased through the reproductive cycle (Figure 3.13B). EPA concentrations in viscera also increased throughout the reproductive cycle, with significantly lower concentrations observed during the spawning period (Figure 3.13B). Within each gonad maturation period, tissue types also differed from one another in ARA and EPA concentrations (Table 3.9). Concentrations were significantly higher in both viscera and skin than in gonad during spawning periods (Figure 3.13), but in post-spawning periods, gonad had higher ARA and EPA concentrations than viscera or skin. In gonad development periods, ARA and EPA concentrations were higher in viscera compared to other tissue types (Figure 3.13).

3.4. Discussion

I found that female *P. californicus* spawned 2 months earlier and contained viscera year-round, compared to neighboring populations in British Columbia, Canada which spawn in late summer and completely reabsorb their viscera in winter months (Cameron and Fankboner 1986). Year round, *P. californicus* from SEAK fed on a variety of items including diatom detritus and terrestrial debris, and FA from these items were incorporated into gonad, skin, and viscera tissues. Data also suggested that lipids stored in skin may be used during egg maturation. Specifically, concentrations of EPA were highest in skin and gonad. When comparing concentrations of ARA and EPA, as well as overall FA composition, among tissue types within each gonad maturation period, gonad and skin were significantly different from viscera.

3.4.1. Temporal Patterns in Reproductive Cycles and Diet

Gonad total lipid concentrations were higher during spawning periods and lower during post-spawning and gonad developing periods. Similar trends have been noted in other studies on sea cucumbers, which linked higher total lipid with larger gonad ratios, and potentially higher reproductive success (Xu et al. 1994, Moran et al. 2013).

Peak spawning period in my study occurred two months earlier (April - May) than in British Columbia, Canada (June - July, Figure 3.1; Cameron and Fankboner 1986). Earlier spawning periods could be due to differences between habitat, inter-annual variability, and/or shifts in spawning seasons over the three decades between studies. Since these populations are separated by only 110 km (1.35° of latitude), differences in the observed timing of spawning are most likely due to changes in the Gulf of Alaska oceanographic conditions over time (Stabeno et al. 2004, Royer and Grosch 2006). It is unlikely that these populations are genetically isolated, due to the prolonged larval periods of *P. californicus* (25 – 107 days; Fankboner and Cameron 1985) and the Alaskan Coastal Current facilitating dispersal of larvae from British Columbian populations to SEAK (Eckert et al. 2007). Warming seawater temperatures could be a factor, with mean increase of 0.12 – 0.25 °C decade^{-1} observed in the Gulf of Alaska; peak temperatures in open ocean surface waters and shallow coastal waters have also occurred earlier in the year over the past 30 years (Cheung et al. 2015). Spawning events for most marine invertebrates are cued by combinations of environmental parameters such as photoperiod, tidal cycles, food, and temperature (Giese 1959, Ramirez 2002), with temperature being a particularly strong cue in holothurians species.

P. californicus in SEAK retained viscera tissue and continued to feed year round, in contrast to other commercially harvested populations in British Columbia, Canada that

completely cease feeding in winter and enter into a dormancy state (Fankboner and Cameron 1985, Fankboner 2002). Year-round feeding in SEAK could be related to changes in regional temperatures and food supplies.

Total lipid in gut content varied significantly between collection dates, but did not correlate with phases of the reproductive cycle, with collection dates in 2012 containing higher lipid content than in 2013. This difference could be due to higher annual primary productivity in 2012 (Strom et al. 2015), which was one of the strongest "cold" PDO years in recent decades (Whitney et al. 2013, Pozo Buil and Di Lorenzo 2015). The Gulf of Alaska experiences shifts in oceanographic conditions in conjunction with "cold" and "warm" years (Stabeno et al. 2004, Sturdevant et al. 2012). During "cold" phases of the Pacific Decadal Oscillation (PDO) the Gulf of Alaska seawater temperatures are colder than neutral PDO phases along the coast, there is a deepening of the mixed layer depth, and increased overall primary production in the region; the opposite is generally true for a "warm" phase PDO (Stabeno et al. 2004). Such shifts in regional oceanographic conditions and increases in diatom abundance have been linked to fluctuations in population size and reproductive potential of other fisheries species, including several species of large crab (Zheng and Kruse 2006) and salmon (Hare et al. 1999).

I found a wide array of material in guts, including diatoms, shell debris, and terrestrial debris (wood matter and pine needles). Deposit-feeding sea cucumbers can be selective omnivores, consuming phytodetritus and benthic diatoms, bacteria, protozoa, and small invertebrates associated with surface sediments (Yingst 1982, Slater and Jeffs 2010). *P. californicus* have peltate oral tentacles, with cauliflower-like structures that can actively select for prey types of different sizes and morphologies (Cameron and Fankboner 1984). Sea cucumbers can also chemically "sense" phytodetritus and bacterial mats, and actively move

towards locations of "fresh" detrital deposits (Hudson et al. 2004); therefore, it is unlikely that my study animals passively consumed particles found in their guts.

FA trophic markers for diatoms (16:4ω1, 16:1ω7, and 16:0), bacteria (14:1 and odd chain FAs), and to a smaller extent terrestrial debris (18:3ω3 and 18:3ω6), were incorporated into all tissue types (FA trophic markers reviewed by Parrish 2013). Bacteria and diatom FA are abundant in all sea cucumber species, suggesting they may be universally important to sea cucumber physiology (David and MacDonald 2002, Hudson et al. 2004, Neto et al. 2006, Xu et al. 2016). However, the bacterial FA may also be produced by microflora associated with the intestinal tract.

Terrestrial FAs may have been directly ingested, or secondarily incorporated through the digestion of bacterial decomposers of the terrestrial debris. Sea cucumbers are unable to directly digest cellulose (Yingst 1982), and no other studies on sea cucumbers have noted the presence of terrestrial debris in gut contents or associated FA markers in tissues. The terrestrial FA markers 18:3ω3 and 18:3ω6 are precursors to DHA and EPA, and these FA can be elongated in some animals (e.g., marine bony fish; Kelly and Scheibling 2012, Parrish 2013). It is unclear if sea cucumbers also have the ability to modify these dietary FA (Monroig et al. 2013). Nonetheless, the abundance of terrestrial debris in gut contents and incorporation of terrestrial FA into sea cucumber tissues suggests that this debris may be an important food source in nearshore areas. Coastal shoreline deforestation in regions of the Northwest Pacific where *P. californicus* are commercially-harvested may thus result in a reduction of terrestrial organic matter input that impacts sea cucumber populations (Blair and Aller 2012, Albert and Schoen 2013).

3.4.2. Temporal Patterns in Tissue Lipid and FA

Sea cucumbers have no dedicated energy storage organ (such as the pyloric caeca in sea stars); therefore, other body tissues may serve this purpose (Lawrence 1976, Oudejans and Van der Sluis 1979). Total lipid and FA storage can occur as triglycerides or as physical tissue mass that can be metabolized to release cell membrane phospholipids. Viscera and gonad ratios were lowest during gonad development periods and highest during spawning and post-spawning periods. Fankboner (2002) suggested that viscera ratios decrease during gonad developing periods (i.e., winter) as a response to changes in salinity, but did not explain why absorption of tissue would occur as a result of osmoregulatory stress. More likely, absorption of visceral tissue mobilizes stored lipids, possibly in part for reproduction. Viscera total lipid content was constant across all collection dates; however, viscera FA composition was distinct from other tissues in post-spawning periods as well as gonad development and spawning periods. Viscera were relatively high in ARA, EPA, and DHA during gonad development and post-spawning periods, but depleted in ARA and EPA during spawning periods. These data collectively may suggest that ARA and EPA from viscera could have been used for egg production and/or tissue growth.

PUFAs, including ARA and EPA, are important in the reproduction of marine invertebrates, providing FA needed for vitellogenesis as well as growth of larvae and adults. Other studies have observed the preferential incorporation of ARA and EPA into reproductive tissues (Hendriks et al. 2003, Ehteshami et al. 2011). EPA, DHA, and ARA are commonly found in the phospholipid cell membranes of cold water species to aid in membrane fluidity (Bell and Sargent 2003), and changes in the amount of these FA in diets could be reflected in gonad ratios (i.e., affecting animals' ability to grow structural and reproductive tissues; Budge et al. 2014). In addition, ARA and EPA are also precursors to prostaglandin, isoprostane, and isofuran

spawning hormones in marine animals, and their abundance in the diet can affect spawning frequency and timing (Parrish 2009).

Tissue ratios and total lipid of structural tissues, including muscle and skin, did not show a clear relationship to reproductive period. Muscle ratios varied with collection dates, but were only slightly higher during gonad development periods than during spawning and post-spawning periods. The lack of seasonal variation in muscle lipid, and low correlation with gonad total lipid and/or gonad ratio, all suggest that muscle is not used for lipid storage for reproduction or growth during low-food periods. It is not surprising that muscle is not used as an energy resource, because *P. californicus* muscle tends to be high in protein and low in lipid (Chang-Lee et al. 1989, Bechtel et al. 2013).

While skin ratios remained relatively constant across the reproductive cycle, skin lipid content varied, suggesting that skin is involved in lipid storage, which has been observed in other sea cucumber species during food limited periods (David and MacDonald 2002). Skin tissue FA composition in females with low gonad ratios was more variable than in those with higher gonad ratios, particularly in PUFAs levels, suggesting a possible mobilization when gonads are developing. The lower variability of total lipid concentrations may suggest that when gonads ratios are high (i.e., spawning periods) FA have already been sequestered from skin. However, I did not find a significant decrease in skin ARA or EPA concentrations across gonad maturation periods. Thus, although skin appears to be storing lipid, it unclear if it is used for gonad development.

3.5. Conclusions

Commercially-harvested populations of *P. californicus* from SEAK are in decline (Clark et al. 2009, Anderson et al. 2011). While the exact causes for these population changes are unknown, dietary shifts may be a contributing factor (Xu et al. 1994, Hendriks et al. 2003). Diatoms and terrestrial debris were important food items for *P. californicus*, and FA trophic markers from these items were incorporated into all tissue types. The relative abundance of diatoms and terrestrial matter in *P. californicus* diets is an important consideration for resource managers, as marine coastal habitats continue to change through costal shoreline deforestation (Schoonmaker 1997, Albert and Schoen 2013) and the oceanographic setting (e.g., PDO; Weingartner et al. 2009, Strom et al. 2015). This information can be used to design effective feed for captive animals in an aquaculture. I also found that tissue ratios, total lipid concentrations, and ARA and EPA concentrations increased in gonads concurrently with decreases in skin, suggesting that skin may be storing energy for gonad maturation. The ability to store energy in skin may allow this species to mitigate shifts in diets as a result of ecosystem changes or seasonal variations in primary production.

3.6. References

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3.7. Figures

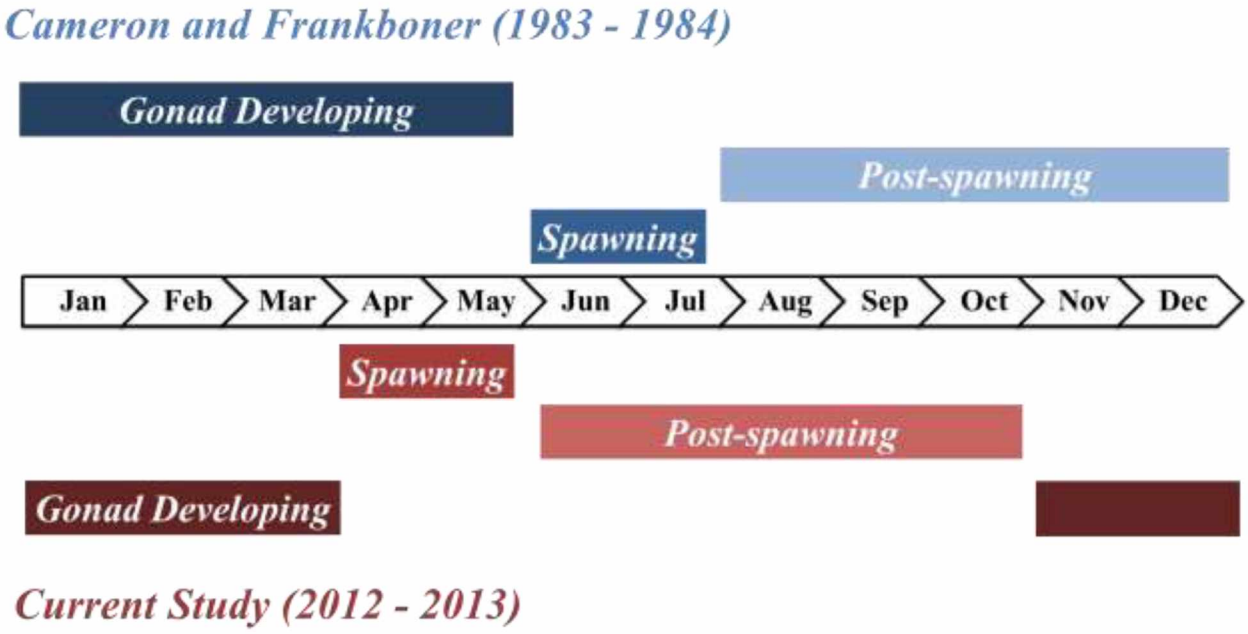

Figure 3.1. Timeline showing collection dates relative to gonad maturation periods. Three periods (spawning, post-spawning, and developing) were identified in this study (SEAK) and in BC, Canada (Cameron and Fankboner (1986). Gonad developing (Nov-11-2012, Dec-11-2012); Spawning (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013); Post-spawning (Aug-26-2012, Jul-24-2013).

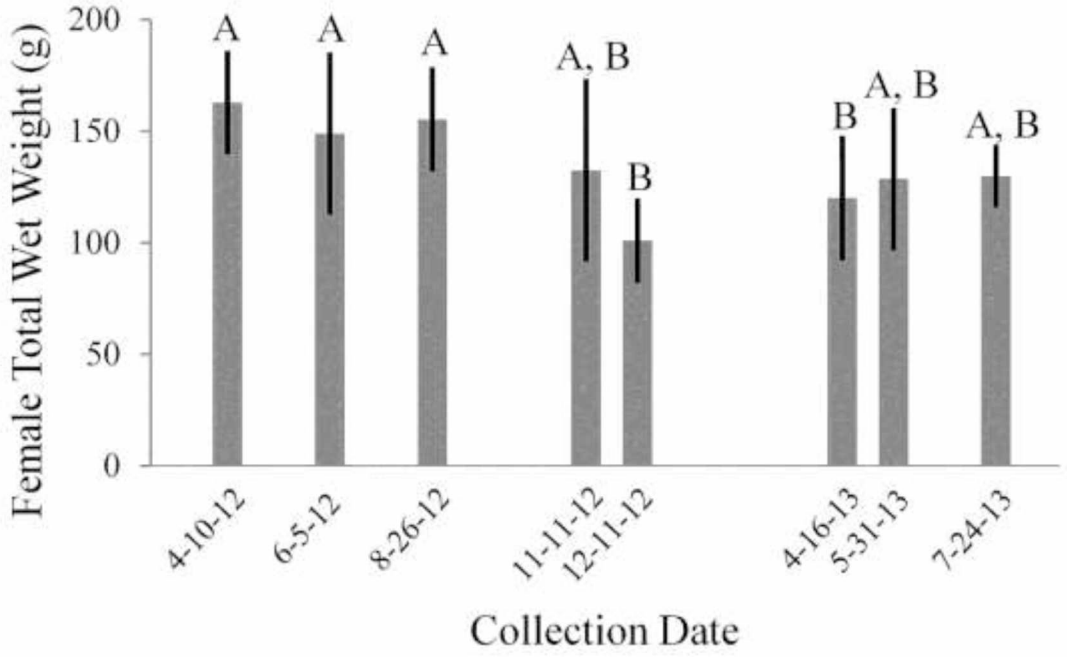

Figure 3.2. Mean total female wet weight by collection date. Bars show ± 1 standard deviation, and letters denote significantly different ($p \leq 0.05$) post-hoc test groupings. N = 10 females per collection date. Gonad developing (Nov-11-2012, Dec-11-2012); Spawning (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013); Post-spawning (Aug-26-2012, Jul-24-2013).

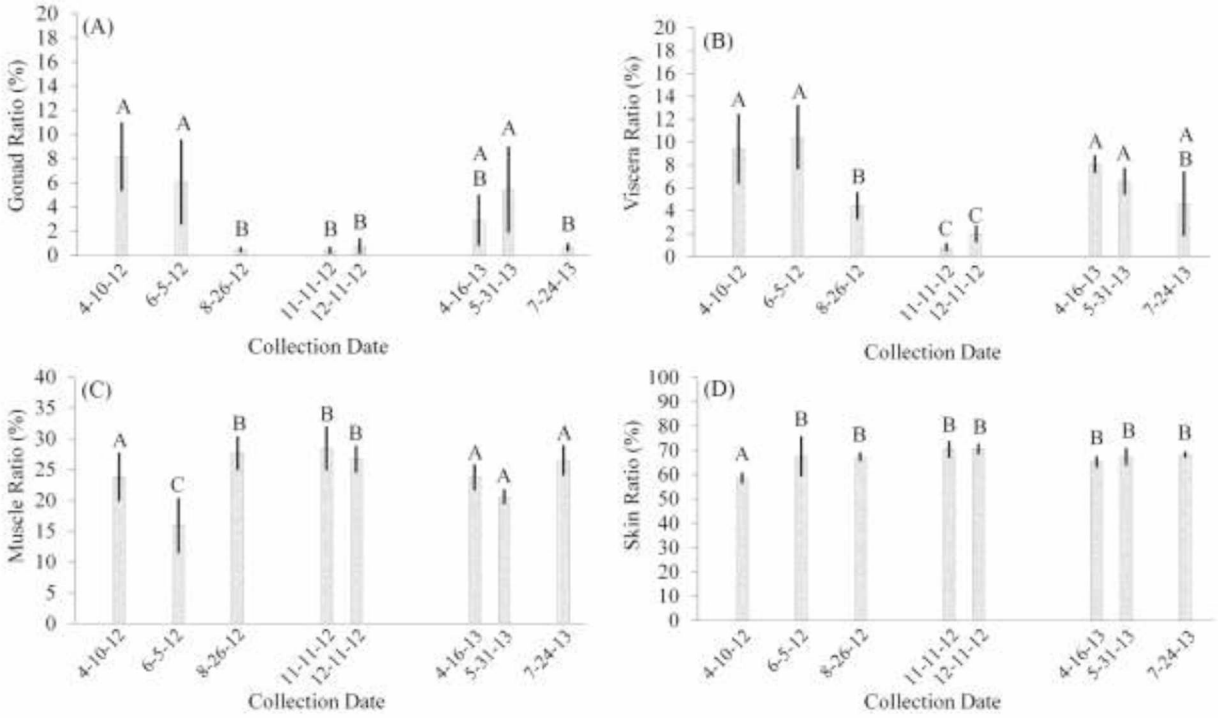

Figure 3.3. Mean tissue ratios by collection dates. (A) gonad, (B) viscera, (C) muscle, (D) skin. Bars show ± 1 standard deviation, and letters denote significantly different ($p \leq 0.05$) post-hoc test groupings among collection dates for each panel. N = 5 females per collection date. Gonad developing (Nov-11-2012, Dec-11-2012); Spawning (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013); Post-spawning (Aug-26-2012, Jul-24-2013).

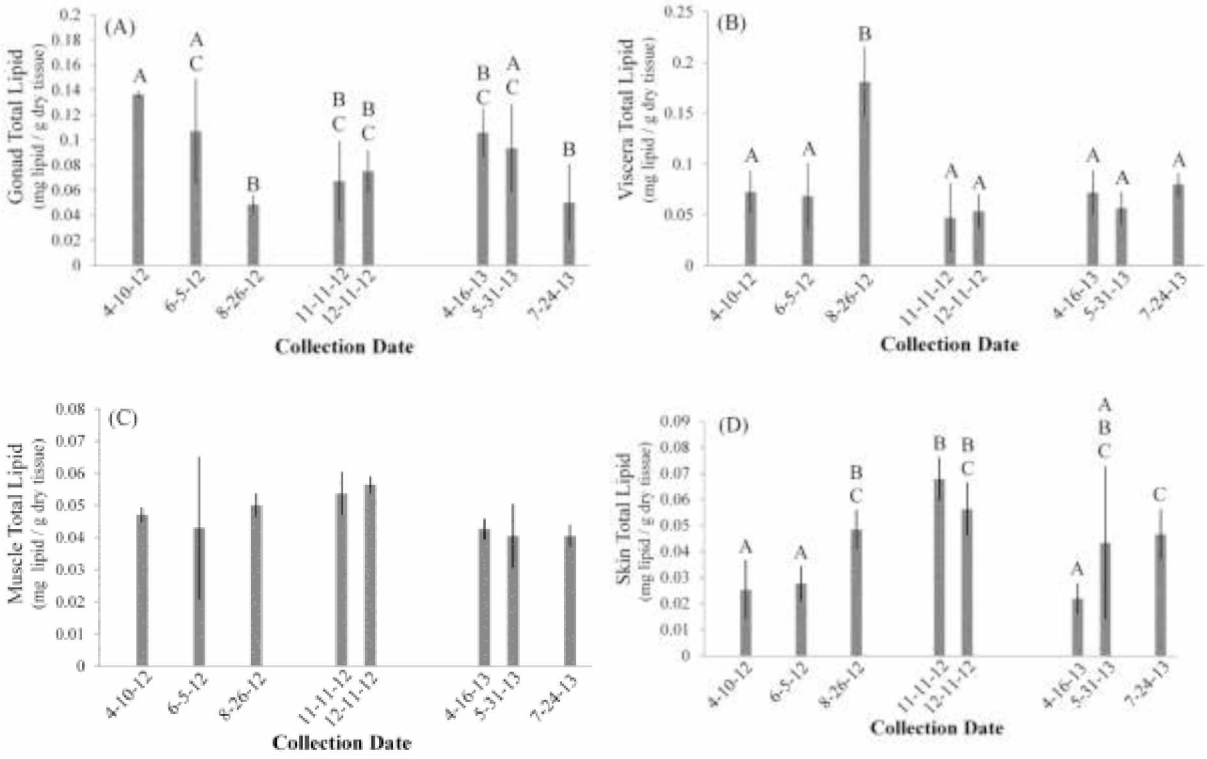

Figure 3.4. Mean total lipids g^{-1} dry tissue by collection dates. (A) gonad, (B) viscera, (C) muscle, (D) skin. Bars show ± 1 standard deviation, and letters denote significantly different ($p \leq 0.05$) post-hoc test groupings of collection dates for each panel. N = 5 females per collection date. Gonad developing (Nov-11-2012, Dec-11-2012); Spawning (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013); Post-spawning (Aug-26-2012, Jul-24-2013).

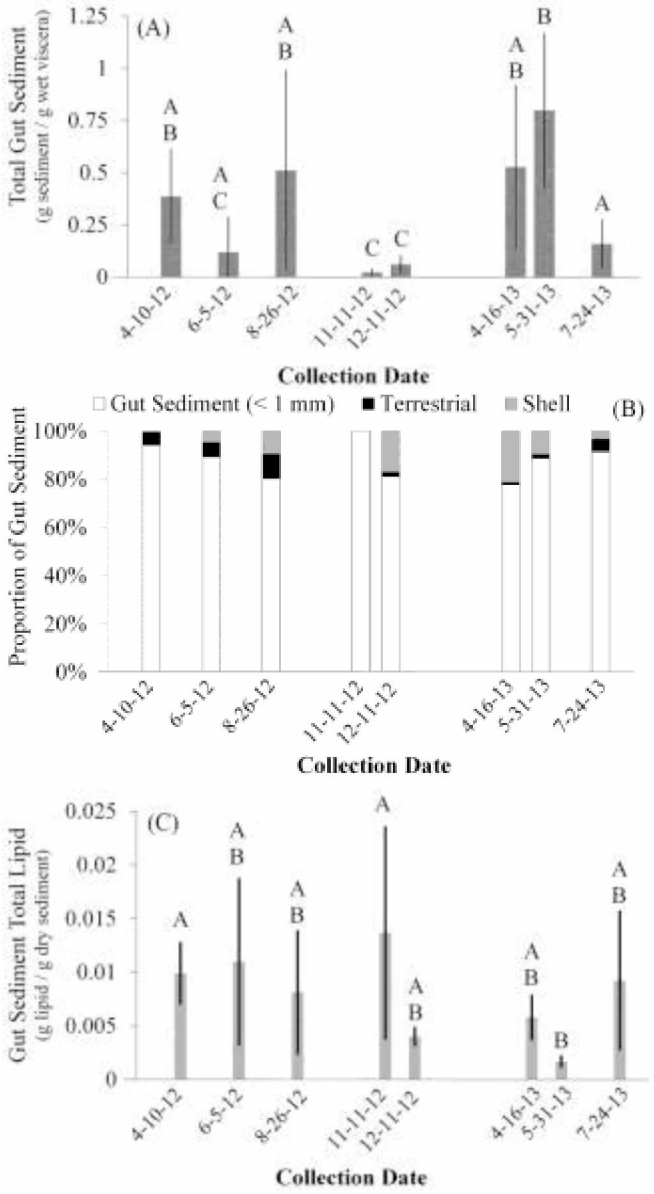

Figure 3.5. Gut content analysis. (A) Mean g gut content g^{-1} total female wet weight ± standard deviations (SD) by collection date. (B) Proportion of gut contents (%) by collection date. (C) Mean mg total lipids g^{-1} dry sediment ± standard deviations by collection date. Letters denote significant ($p \leq 0.05$) post-hoc groupings of collection dates for panels (A) and (C). N = 5 females per collection date. Gonad developing (Nov-11-2012, Dec-11-2012); Spawning (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013); Post-spawning (Aug-26-2012, Jul-24-2013).

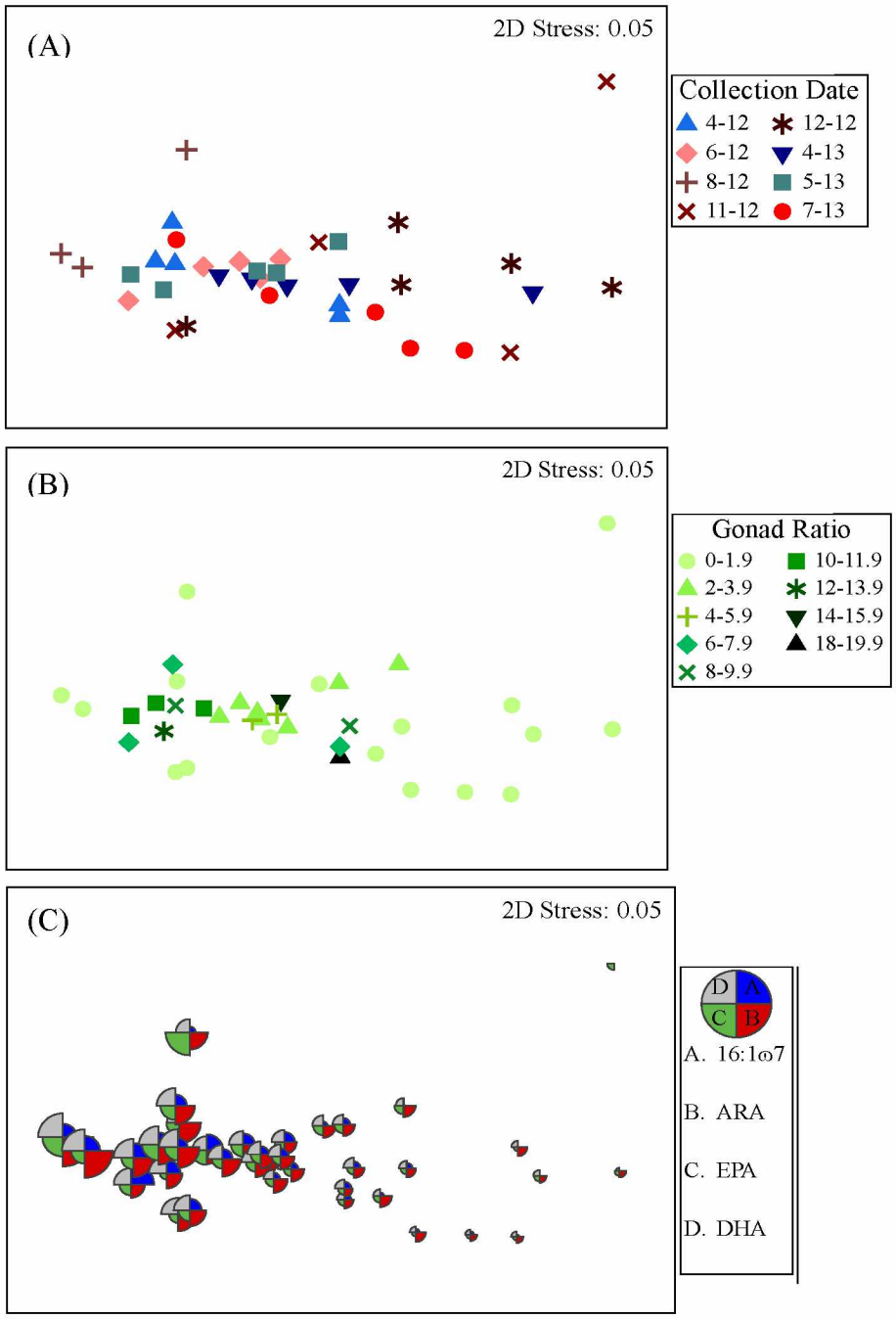

Figure 3.6. Gonad FA non-metric MDS. (A) plotted by collection date, blue symbols denote spawning periods (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013), red symbols denote post-spawning (Aug-26-2012, Jul-24-2013) and gonad development (Nov-11-2012, Dec-11-2012) periods; (B) plotted by gonad ratios; (C) showing relative amounts of FA contributing

most to total SIMPER group similarity shown in Table 3.1; wedge size a denotes the range in the abundance of that given FA in sample. No significant SIMPROF groupings were found.

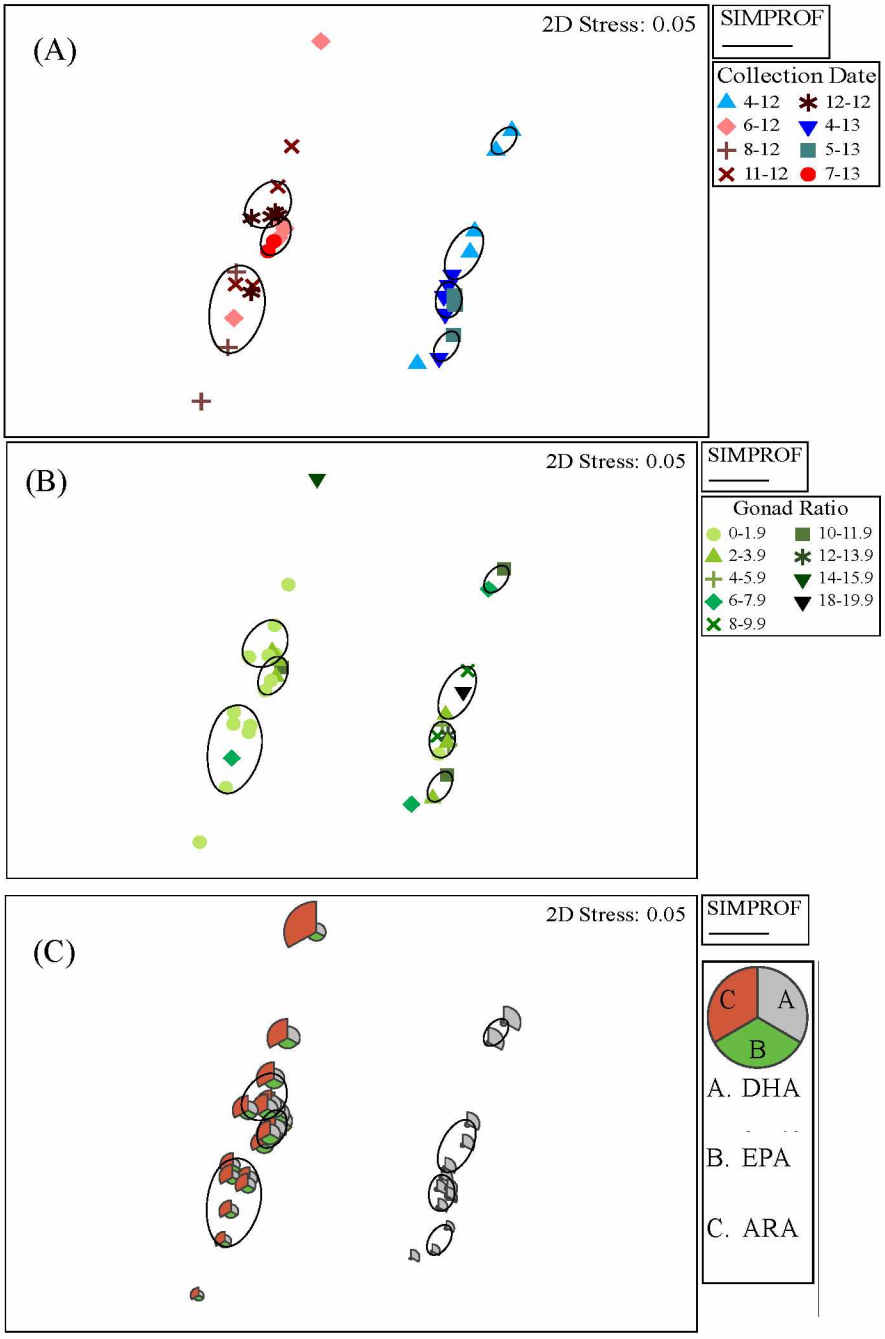

Figure 3.7. Viscera FA non-metric MDS. (A) plotted by collection date, blue symbols denote spawning periods (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013), red symbols denote post-spawning (Aug-26-2012, Jul-24-2013) and gonad development (Nov-11-2012, Dec-11-2012) periods; (B) plotted by gonad ratios; (C) showing relative amounts of FA contributing

most to total SIMPER group similarity shown in Table 3.2; wedge size a denotes the range in the abundance of that given FA in sample. Circles indicate significant SIMPROF groupings.

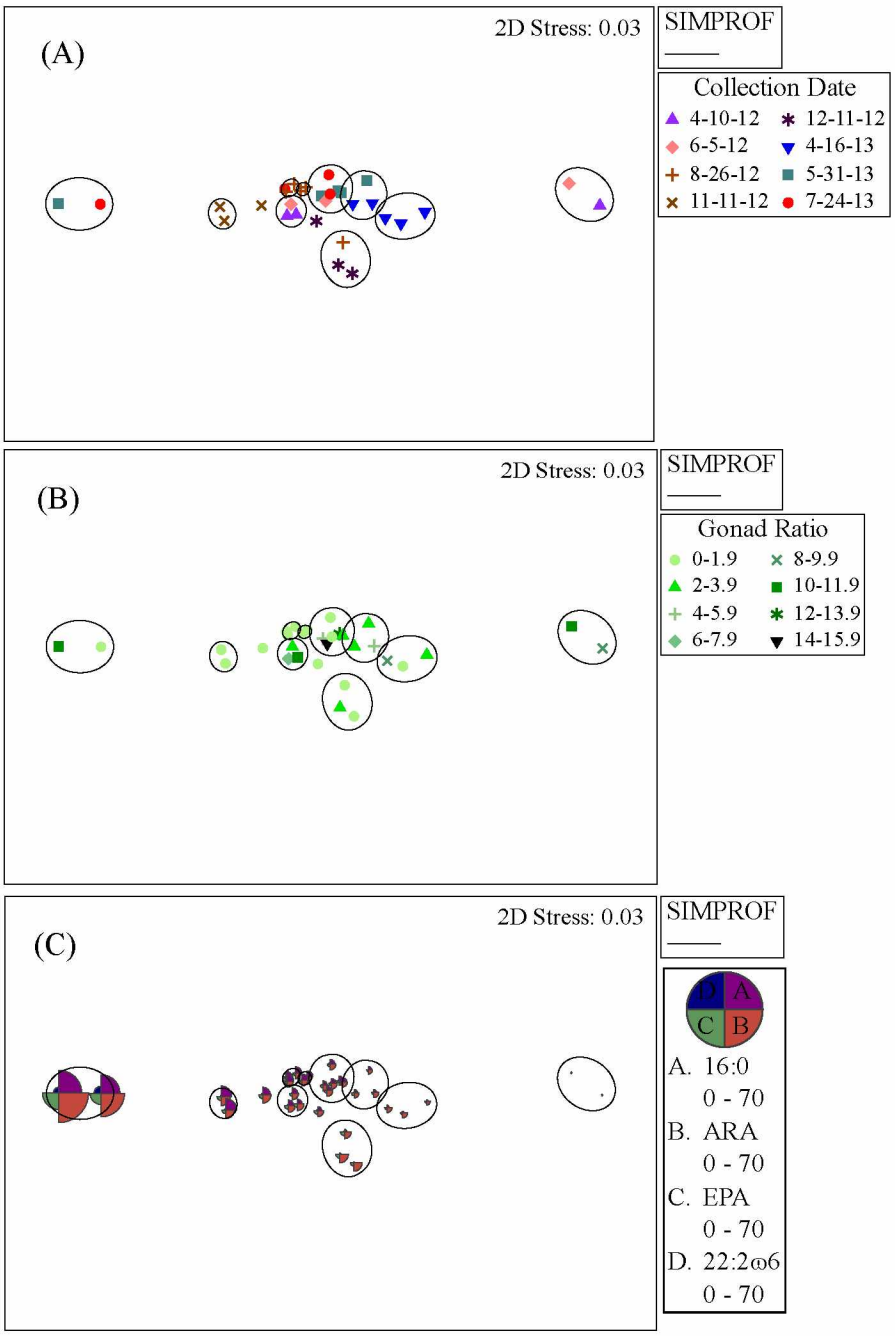

Figure 3.8. Skin non-metric MDS. (A) plotted by collection date, blue symbols denote spawning periods (Apr-10-2012, Jun-5-2012, Apr-16-2013, May-31-2013), red symbols denote post-spawning (Aug-26-2012, Jul-24-2013) and gonad development (Nov-11-2012, Dec-11-2012) periods; (B) plotted by gonad ratios; (C) showing relative amounts of FA contributing most to

total SIMPER group similarity shown in Table 3.3; wedge size a denotes the range in the abundance of that given FA in sample. Circles indicate significant SIMPROF groupings.

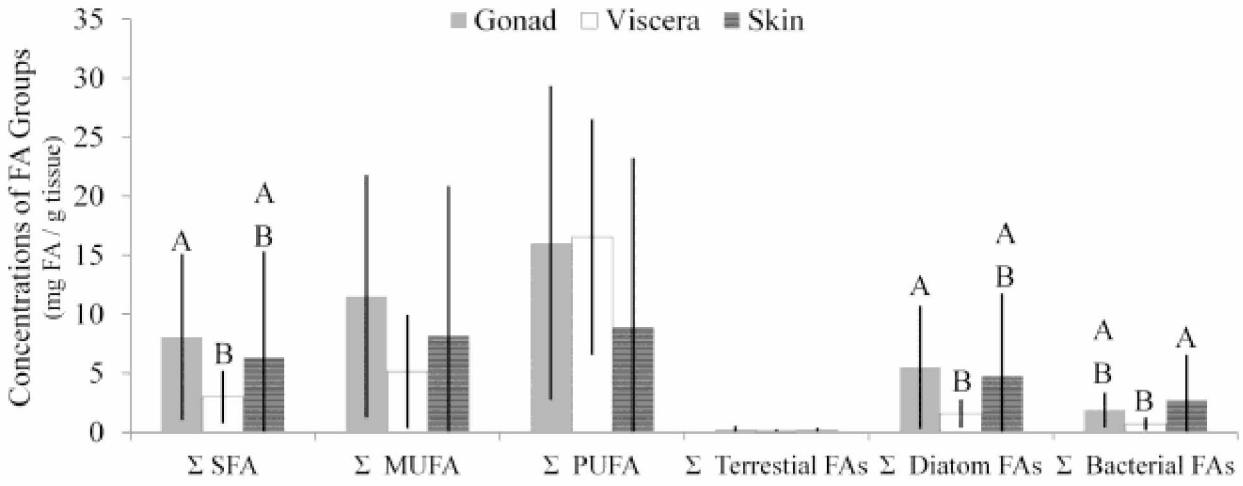

Figure 3.9. Mean concentrations of Σ SFA, Σ MUFA, Σ PUFA, Σ Terrestrial FA, Σ Diatom FA, Σ Bacterial FA. Σ Terrestrial FA comprise 18:3ω3 and 18:3ω6, Σ Diatom FA comprise 16:1ω7, 16:4ω1, and 16:0, and Σ Bacterial FA comprise 14:1 and odd C chained FA.Bars show ± 1 standard deviation, and letters denote post-hoc testing for significant differences between tissue types in a single FA group. N = 5 females per tissue type.

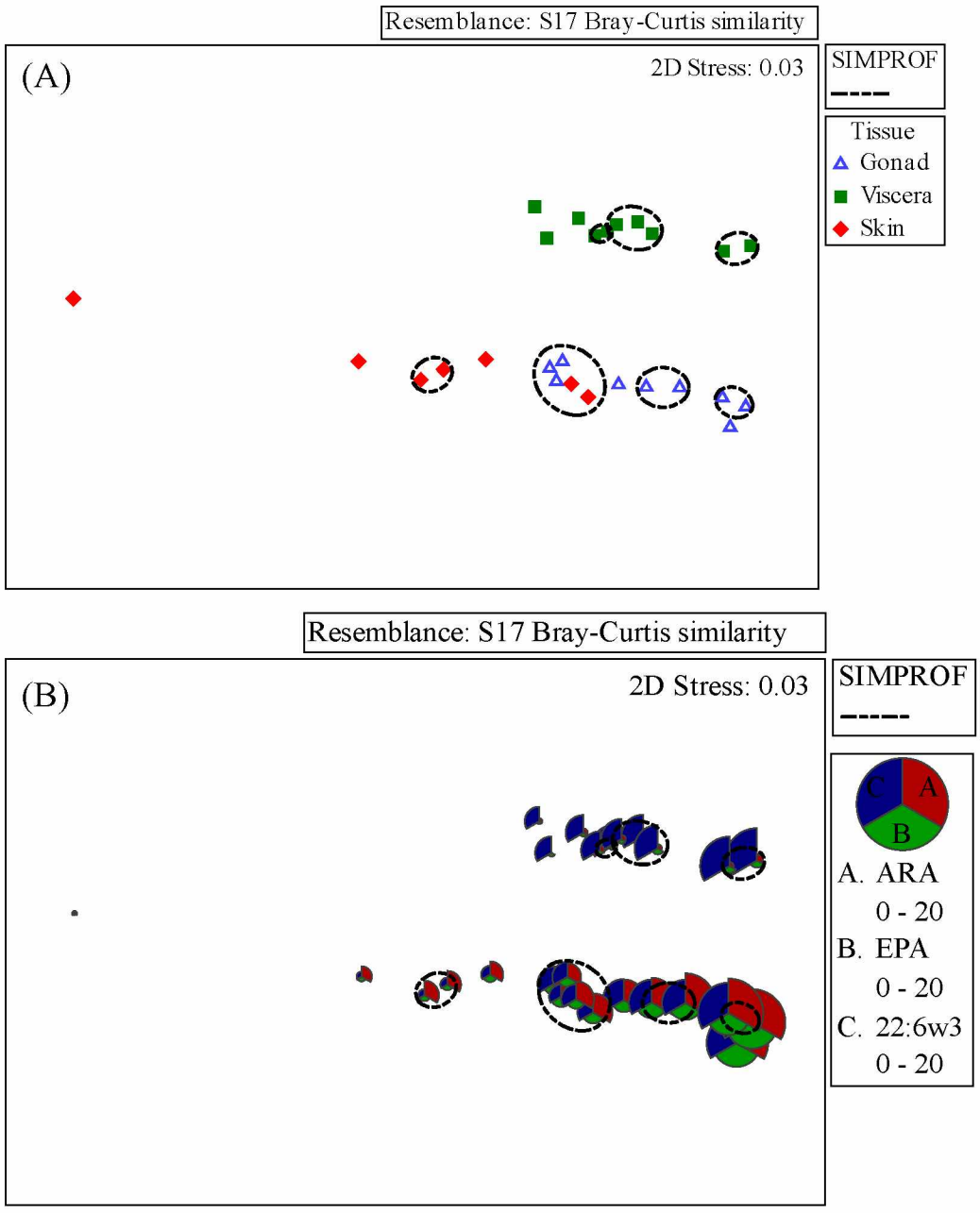

Figure 3.10. Spawning period FA non-metric MDS and SIMPER similarity contributions. Non-metric MDS (A) plotted differences in FA composition among tissue types (gonad, viscera, and skin) during the spawning period; (B) concentrations of FA contributing most to total SIMPER group similarity shown in Table 3.5. Wedge size and values in the legend denotes the range in the abundance of that given FA in sample. Circles denote significant SIMPROF groupings.

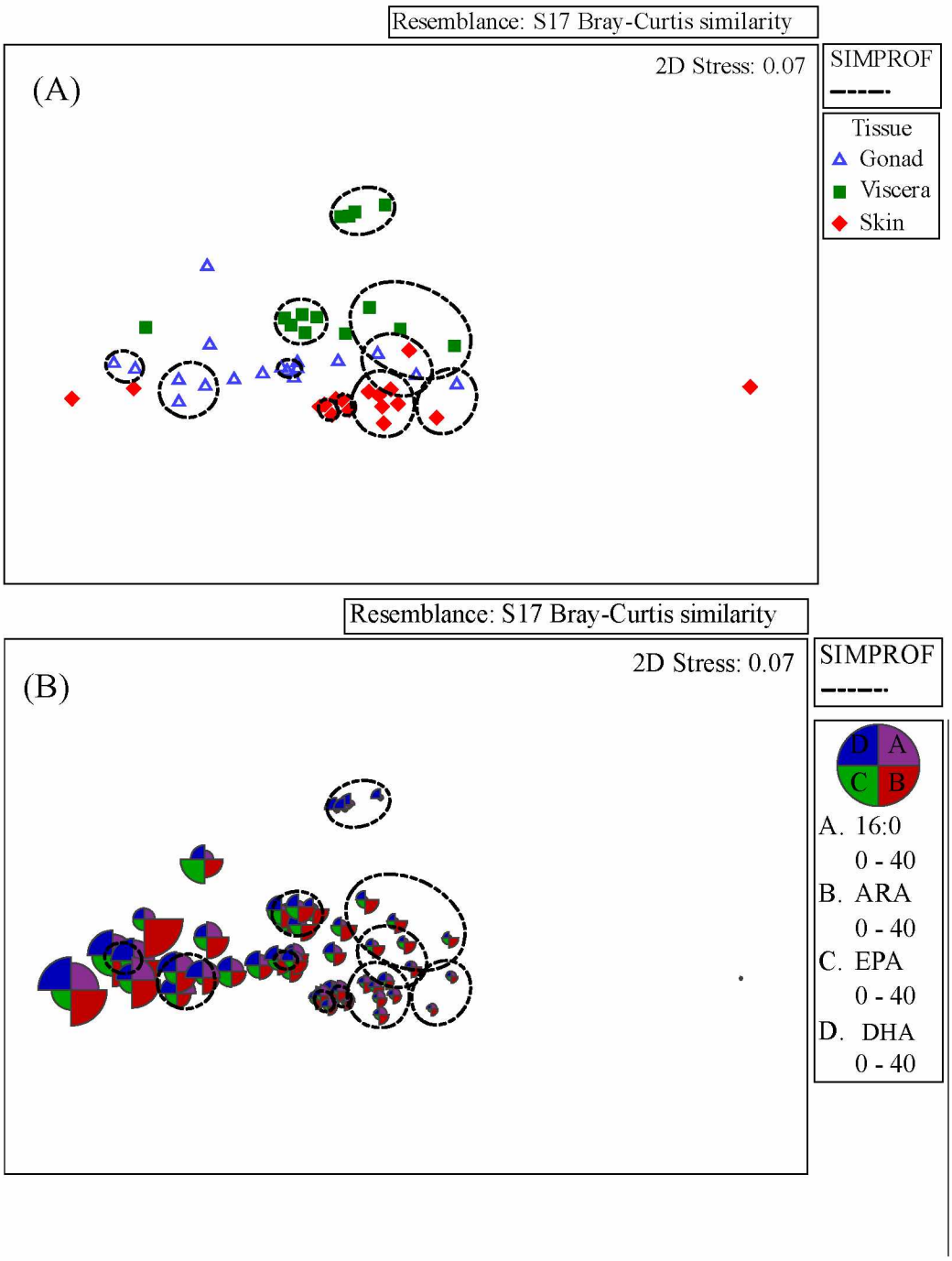

Figure 3.11. Post-spawning period FA non-metric MDS and SIMPER similarity contributions. Non-metric MDS (A) plotted differences in FA composition among tissue types (gonad, viscera, and skin) during the post-spawning period; (B) concentrations of FA contributing most to total SIMPER group similarity shown in Table 3.6. Wedge size denotes the relative concentration of that each FA in sample. Circles denote significant SIMPROF groupings.

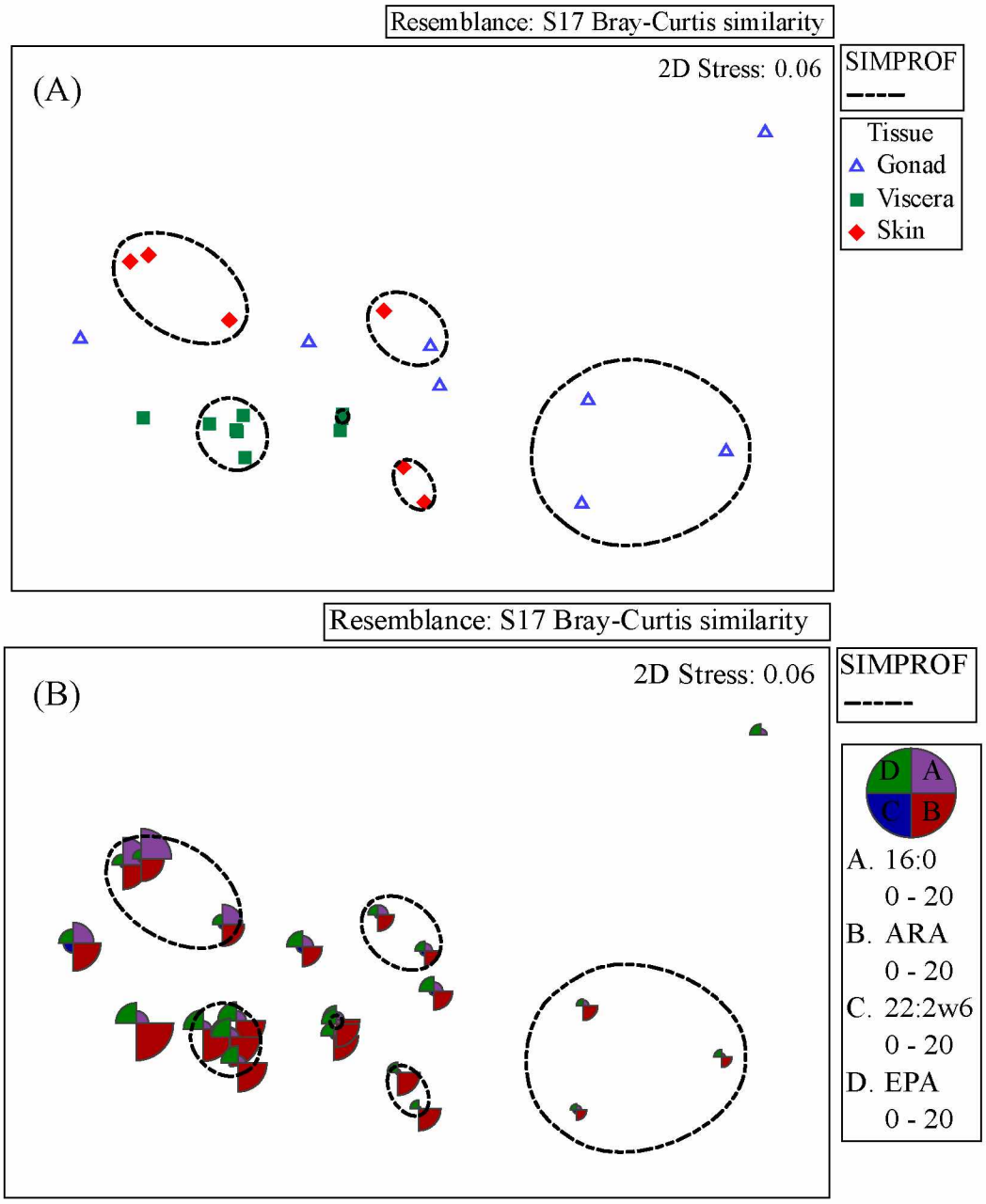

Figure 3.12. Developing period FA non-metric MDS and SIMPER similarity contributions. Non-metric MDS (A) plotted differences in FA composition among tissue types (gonad, viscera, and skin) during the gonad development period; (B) concentrations of FA contributing most to total SIMPER group similarity shown in Table 3.7. Wedge size denotes the concentration of each FA in sample Circles denote significant SIMPROF groupings.

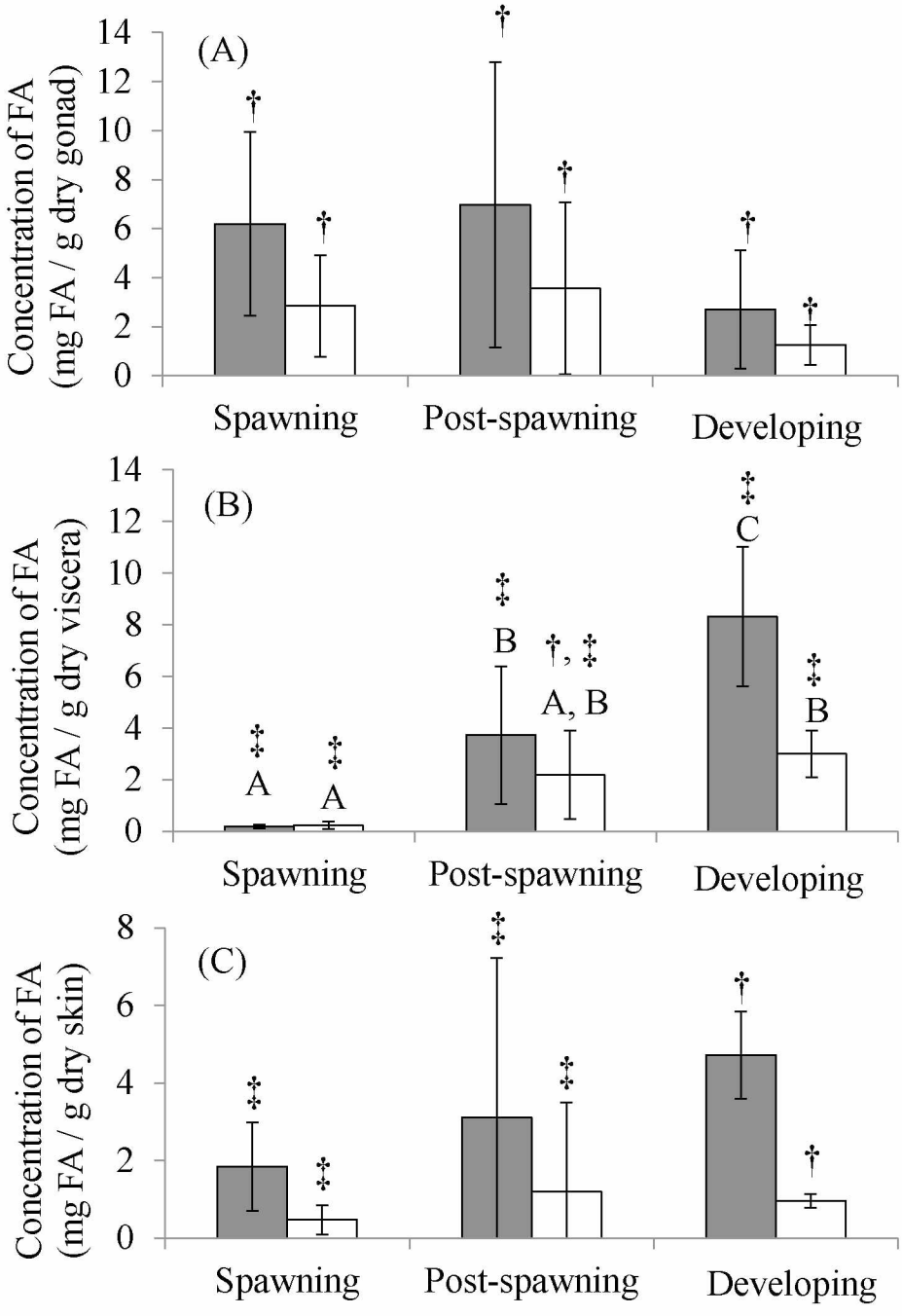

Figure 3.13. Mean concentrations of ARA and EPA. Black lines denote ± standard deviation, gray bars show ARA (20:4ω6) and white bars show EPA (20:5ω3) by tissue type; gonad (A), viscera (B), skin (C). Letters denote post-hoc testing for significant differences between gonad

maturation periods for each tissue type (i.e., within a figure panel). Symbols denote post-hoc testing for significant differences between gonad maturation periods (i.e., among figure panels).

3.8. Tables

Table 3.1. SIMPER analysis of gonad FA composition by collection date. Table shows FA accounting for at least 50 % of within-group similarity.

Sample	FA	Individual Contribution to Total Similarity (%)
Spawning *(Apr-10-2012)* (56.03 % mean similarity)	20:4ω6 (ARA)	15.74
	22:6ω3 (DHA)	13.83
	16:0	8.42
	20:5ω3 (EPA)	7.89
	16:1ω7	6.86
Post spawning *(Jun-5-2012)* (67.93 % mean similarity)	22:6ω3 (DHA)	15.34
	20:4ω6 (ARA)	15.15
	16:1ω7	11.73
	20:5ω3 (EPA)	8.95
Post spawning *(Aug-26-2012)* (56.03 % mean similarity)	20:4ω6 (ARA)	20.46
	22:6ω3 (DHA)	12.49
	20:5ω3 (EPA)	11.52
	16:0	7.68
Gonad developing *(Nov-11-2012)* (22.09 % mean similarity)	20:5ω3 (EPA)	24.38
	20:4ω6 (ARA)	15.28
	16:0	11.95
Gonad developing *(Dec-11-2012)* (39.51 % mean similarity)	20:4ω6 (ARA)	38.79
	22:6ω3 (DHA)	16.56
Spawning *(Apr-16-2013)* (52.71 % mean similarity)	20:4ω6 (ARA)	20.40
	22:6ω3 (DHA)	19.52
	20:5ω3 (EPA)	9.32
	16:0	7.51
	16:1ω7	4.98
Post spawning *(May-31-2013)* (60.70 % mean similarity)	20:4ω6 (ARA)	18.79
	22:6ω3 (DHA)	16.03
	16:0	9.53
	20:5ω3 (EPA)	7.42
Post spawning *(Jul-24-2013)* (49.53 % mean similarity)	20:4ω6 (ARA)	22.07
	22:6ω3 (DHA)	9.65
	16:0	8.45
	20:5ω3 (EPA)	6.47
	24:1ω9	6.12

Table 3.2. SIMPER analysis of viscera FA composition by gonad spawning periods and collection date. Table shows FA accounting for at least 50 % of within-group similarity.

Sample	FA	Individual Contribution to Total Similarity (%)
Spawning *(Apr-10-2012)* (61.00 % mean similarity)	20:3ω6	23.84
	20:4ω3	19.20
	22:6ω3 (DHA)	18.78
Post spawning *(Jun-5-2012)* (58.79 % mean similarity)	20:4ω6 (ARA)	30.75
	22:6ω3 (DHA)	16.50
	20:5ω3 (EPA)	14.43
Post spawning *(Aug-26-2012)* (66.54 % mean similarity)	20:4ω6 (ARA)	36.38
	20:5ω3 (EPA)	17.38
	22:6ω3 (DHA)	13.07
Gonad developing *(Nov-11-2012)* (64.48 % mean similarity)	20:4ω6 (ARA)	37.70
	20:5ω3 (EPA)	13.24
	22:6ω3 (DHA)	12.02
Gonad developing *(Dec-11-2012)* (79.40 % mean similarity)	20:4ω6 (ARA)	35.58
	20:5ω3 (EPA)	14.03
	22:6ω3 (DHA)	14.01
Spawning *(Apr-16-2013)* (81.51 % mean similarity)	20:3ω6	28.04
	22:6ω3 (DHA)	19.14
	20:4ω3	14.08
Post spawning *(May-31-2013)* (85.15 % mean similarity)	20:3ω6	31.47
	22:6ω3 (DHA)	16.52
	20:4ω3	14.56
Post spawning *(Jul-24-2013)* (90.17 % mean similarity)	20:4ω6 (ARA)	29.72
	20:5ω3 (EPA)	15.83
	22:6ω3 (DHA)	13.71

Table 3.3. SIMPER analysis of skin FA composition by gonad spawning periods and collection date. Table shows FA accounting for at least 50 % of within-group similarity.

Sample	FA	Individual Contribution to Total Similarity (%)
Spawning *(Apr-10-2012)* (29.58 % mean similarity)	20:4ω6 (ARA)	18.63
	22:6ω3 (DHA)	13.14
	20:3ω6	9.60
	16:0	7.71
	16:1ω7	5.58
Post spawning *(Jun-5-2012)* (58.79 % mean similarity)	20:4ω6 (ARA)	18.40
	16:1ω7	13.38
	16:0	9.33
	22:6ω3(DHA)	7.34
	18:0	5.71
Post spawning *(Aug-26-2012)* (66.54 % mean similarity)	20:4ω6 (ARA)	15.92
	16:0	12.14
	22:6ω3 (DHA)	8.17
	16:1ω7	6.88
	18:0	4.84
Gonad developing *(Nov-11-2012)* (64.48 % mean similarity)	16:0	37.70
	20:4ω6 (ARA)	13.24
	16:1ω7	12.02
Gonad developing *(Dec-11-2012)* (79.40 % mean similarity)	20:4ω6 (ARA)	47.64
	20:5ω3 (EPA)	11.07
Spawning *(Apr-16-2013)* (73.06 % mean similarity)	20:4ω6 (ARA)	32.27
	22:6ω3 (DHA)	6.51
	20:5ω3 (EPA)	5.77
	16:0	5.43
	18:0	4.95
Post spawning *(May-31-2013)* (50.16 % mean similarity)	20:4ω6 (ARA)	15.52
	16:0	11.68
	16:1ω7	9.59
	22:6ω3 (DHA)	7.75
	18:0	5.98
Post spawning *(Jul-24-2013)* (91.71 % mean similarity)	20:4ω6 (ARA)	19.94
	16:0	14.14
	16:1ω7	12.12
	22:6ω3	5.70

Table 3.4. Results of one-way ANOVAs for differences in Σ SFA, Σ MUFA, Σ PUFA, Σ Terrestrial FA biomarkers, Σ Diatom FA biomarkers, Σ Bacterial FA biomarkers among tissue types. Specific tissue type are skin, gonad, and muscle, and analysis is regardless of collection date. * denotes significance after Bonferroni correction for multiple testing.

FA	F value, p value
Σ SFA	F = 5.756, p = 0.004 *
Σ MUFA	F = 4.125, p = 0.019 *
Σ PUFA	F = 3.773, p = 0.026 *
Σ Terrestrial FA	F = 2.465, p = 0.089
Σ Diatom FA	F = 6.700, p = 0.002 *
Σ Bacterial FA	F = 6.803, p = 0.002 *

Table 3.5. SIMPER analysis of FA composition between tissue types showing dissimilarities for the spawning gonad maturation period. Tissue types are skin, gonad, and viscera. Table shows FA accounting for at least 50 % of between-tissue dissimilarity.

Samples	FA	Individual Contribution to Total Dissimilarity (%)
Gonad and Viscera (61.75% mean dissimilarity)	20:3ω6	17.39
	20:4ω6	14.85
	20:4ω3	10.64
	22:6ω3 (DHA)	7.39
Gonad and Skin (62.59% mean dissimilarity)	22:6ω3 (DHA)	14.87
	20:4ω6 (ARA)	14.44
	20:5ω3 (EPA)	7.44
	16:0	6.53
	16:1ω7	5.58
Viscera and Skin (72.66% mean dissimilarity)	20:3ω6	26.21
	20:4ω6 (ARA)	15.55
	22:6ω3 (DHA)	14.69

Table 3.6. SIMPER analysis of FA composition between tissue showing dissimilarities for the post-spawning gonad maturation period. Tissue types are types skin, gonad, and viscera. Table shows FA accounting for at least 50 % of between-tissue dissimilarity.

Samples	FA	Individual Contribution to Total Dissimilarity (%)
Gonad and Viscera (57.19 % mean dissimilarity)	20:4ω6 (ARA) 22:6ω3 (DHA) 16:0 20:5ω3 (EPA) 16:1ω7	14.00 7.88 7.58 7.10 5.82
Gonad and Skin (57.02 % mean dissimilarity)	20:4ω6 (ARA) 22:6ω3 (DHA) 16:0 20:5ω3 (EPA) 16:1ω7	13.18 11.07 8.03 7.82 7.06
Viscera and Skin (60.85 % mean dissimilarity)	20:4ω6 (ARA) 22:6ω3 (DHA) 20:5ω3 (EPA) 20:3ω6 16:0	17.73 9.45 8.30 8.13 7.13

Table 3.7. SIMPER analysis of FA composition between tissue types showing dissimilarities for the developing gonad maturation period. Tissue types are types skin, gonad, and viscera. Table shows FA accounting for at least 50 % of between-tissue dissimilarity.

Samples	FA	Individual Contribution to Total Dissimilarity (%)
Gonad and Viscera (60.83 % mean dissimilarity)	20:4ω6 (ARA)	27.93
	22:6ω3 (DHA)	10.42
	20:5ω3 (EPA)	8.65
Gonad and Skin (63.22 % mean dissimilarity)	20:4ω6 (ARA)	16.36
	16:0	10.90
	16:1ω7	5.98
	22:6ω3 (DHA)	5.54
	18:0	5.43
Viscera and Skin (48.27 % mean dissimilarity)	20:4ω6 (ARA)	14.83
	16:0	10.80
	22:6ω3 (DHA)	8.67
	20:5ω3 (EPA)	8.57
	16:1ω7	5.84

Table 3.8. ANOVA table for individual ANOVA testing ARA and EPA concentrations for each tissue type among gonad maturation periods. Tissue types are gonad, viscera, and skin, and maturation periods are spawning, post-spawning, and developing. * denotes significant differences among gonad maturation periods.

Tissue Type	ARA (20:4ω6)	EPA (20:5ω3)
Gonad	F = 2.26, p = 0.121	F = 1.90, p = 0.167
Viscera	F = 31.23, p = 0.004 *	F = 13.30, p = 0.007 *
Skin	F = 11.27, p = 0.296	F = 0.41, p = 0.667

Table 3.9. ANOVA table for individual ANOVA testing ARA and EPA concentrations by gonad maturation periods among tissue types. Maturation periods are spawning, post-spawning, and developing, and tissue types are gonad, viscera, and skin.* denotes significance among tissue types.

Gonad Maturation Period	ARA (20:4ω6)	EPA (20:5ω3)
Spawning	$F = 17.10$, $p \leq 0.001$*	$F = 12.23$, $p = 0.002$ *
Post-spawning	$F = 3.64$, $p = 0.034$ *	$F = 3.41$, $p = 0.042$ *
Developing	$F = 13.01$, $p = 0.002$ *	$F = 17.26$, $p \leq 0.001$*

Summary and Conclusions

My research addressed the effects of maternal diet, particularly the importance of fatty acid (FA) composition, on reproductive fitness of deposit feeders using the commercially harvested California sea cucumber *(Parastichopus californicus)* as a model organism. Marine organisms rely on FA diversity to maintain functional food webs (Hooper et al. 2005, Kelly and Scheibling 2012, Parrish 2013). However, not all marine invertebrates are capable of *de-novo* synthesizing the FA required for growth and reproduction; therefore, they must obtain them from primary producers through their diets (Parrish 2009). FA are important not only for cell membrane formation, maintenance, and energy pathways, but also in the formation of hormone-like compounds (i.e., eicosanoids) that are used for signaling reproductive processes and growth (Brett and Muller-Navarra 1997, Parrish 2009).

Reproductive strategies, including timing of spawning, sex ratios, fecundity, and gametogenesis have important effects on population structure, but data for *P. californicus* have been limited to Washington State and British Columbia, Canada populations from the 1980s (Strathmann and Sato 1969, Cameron and Fankboner 1986, Smiley 1988, Cameron and Fankboner 1989). With the rising international demand for sea cucumber products (Anderson et al. 2011), these data must be expanded to include other regions to better manage fisheries and expand aquaculture production of this species in the Northwest Pacific.

There are currently four main fishing regions for *P. californicus*: Kodiak AK, Southeast AK, Washington State, and British Columbia. In Chapter 1, I observed peak spawning for *P. californicus* in Southeast AK from April to June, about two months earlier than previously observed in British Columbia in the 1980s. I also found that gonad indices (GI) were lower in Southeast AK than in British Columbia, indicating that reproductive potential may vary between

geographic locations and/or over time. GI from Southeast Alaska were only moderately correlated with gonad lipid and gonad maturation period, suggesting that GI may not be an ideal indicator for the timing of spawning; similar results were also reported for purple sea urchin populations from California (Conor 1972, Ebert et al. 2011). The differences in GI between the Southeast AK population and the neighboring British Columbia population may mean that each of these fishing regions needs to be independently monitored and managed.

In Chapter 1, I also described a novel live-spawning method that I developed for use in experimental aspects of this study. Live spawning yielded significantly higher estimates of fecundity and viable eggs than strip spawning methods, demonstrating that live spawning is both feasible and an improvement to currently available strip-spawning methods (Strathmann and Sato 1969, Chen et al. 1991). These were also the first estimates of *P. californicus* fecundity based on live-spawning, and represent more accurate estimates for use in population models that are currently being developed for Alaska by the Alaska Department of Fish and Game (ADF&G). Fecundity estimates may also be combined with genetic population data that are being collected by ADF&G and University of Washington to examine connectivity between fishing regions via larval dispersal, by understanding how many larvae enter into a system by each female.

Reproductive fitness is directly affected by body condition, which is in turn affected by the consumption of FA and other lipids (Lester et al. 2007, Poorbagher et al. 2010). In Chapter 2, I conducted controlled feeding experiments using two microalgal diets with different nutritional profiles. Body- and egg-condition variables were measured in females that were fed one of two diets differing in nutritional and FA composition. Subsequent timing of larval development and survival were recorded for pre- and post-feeding stages. FA analyses were also

conducted on feeds, spawned gonads, and eggs to identify specific FAs allocated to reproduction. I examined the effects of phytodetritus composition on the reproductive fitness of a deposit-feeding sea cucumber (*Parastichopus californicus*) through captive feeding experiments. Experimental results suggested that *P. californicus* populations exhibit a possible trade-off between the number of eggs produced by each female and the fitness of larvae that develop from those eggs.

Although female body condition and egg size were similar between feed treatments, females fed the green alga *Tetraselmis* sp. (TS) had higher fecundity than those fed the diatom *Thalassiosira* sp. (TW); however, while larval development rates were similar, TS larvae had slightly lower survival. Such reproductive plasticity can increase overall population fitness when there are adequate environmental cues, such as food quantity, which can trigger changes in reproductive strategies (Szuwalski and Hollowed 2016). If environmental conditions are favorable to larvae, then producing a larger number of less energy-dense eggs (such as in the TS feed treatment) would be beneficial to the population, because planktotrophic larvae could feed on phytoplankton. In contrast, if larval food is less abundant, producing a smaller number of more energy-dense eggs (such as in the TW feed treatment) would be favored, because larvae would be better equipped with energy resources derived from the egg. Examples of oceanographic conditions that could result in these shifts include regional warming and freshening of surface waters, which favors small phytoplankton taxa such as green algae and flagellates over diatoms (Laws et al. 1988, Morán et al. 2010, Chavez et al. 2011).

In Chapter 2, I found significant differences in the abundance of the essential FA ARA, EPA, and DHA in spawned gonads, and the saturated FA 12:0, 16:0, and 18:0 in eggs of females given the different experimental feed treatments. Essential FA must be acquired from food, and

serve specific roles within organisms (Brown et al. 1997). These polyunsaturated FA (PUFA) increase membrane fluidity, especially in cold water environments, and facilitate cellular energy production by increasing the speed of the membrane-bound electron transport chain (Lingwood and Simons 2010). High levels of ARA, EPA, and DHA in adult diets result in the production of larger, faster-growing larvae with better survival rates in shrimp and oysters (Xu et al. 1994, Hendriks et al. 2003). In contrast, short-chain saturated FA (SFA), including 12:0, 16:0 and 18:0, have been correlated with larval growth and survival rates (Goedkoop et al. 2007), possibly because energy stored as SFAs is released more quickly and efficiently than in more complex longer carbon chains with higher degrees of saturation. The relative abundance of these PUFAs and SFAs in marine invertebrate diets may affect egg quality and larval fitness, and thus recruitment success. Nonetheless, further studies are needed to determine to what extent longer term (i.e., multiple reproductive cycles) FA limitation will have on population stability.

Drastic shifts are occurring in benthic coastal primary producers with a changing climate in the North Pacific region, from shifts in dominance by seagrass, kelps, and diatoms to green macro- and microalgae (Valiela et al. 1997, Touchette et al. 2007, Chavez et al. 2011). These shifts are predicted to cause shortages of specific FA, including EPA, ARA, and 18:1ω9 (Kelly and Scheibling 2012). Nonetheless, it is difficult to predict how changes in food supplies will in fact affect regional populations without baseline data from the current *in situ* population. In chapter 3, I analyzed gut contents of wild populations from the Southeast AK fishery area, and found that diatoms and terrestrial debris were important components of the *P. californicus* diet, and that EPA and other trophic markers for these dietary items (including 16:1ω7 and ARA) were incorporated into all sea cucumber tissue types. The appearance of diatoms and terrestrial debris in the diet is an important consideration for regional managers, as habitats change due to

coastal deforestation and costal erosion (Schoonmaker 1997, Albert and Schoen 2013) and climate oscillations (i.e., PDO shifts; Weingartner et al. 2009, Strom et al. 2015). Although shifts in primary production are predicted to directly affect FA diversity, and in turn ecosystem functioning (Kelly and Scheibling 2012, Parrish 2013), data are lacking on the specific roles of many FA in animals, particularly marine invertebrates.

Marine invertebrates could mitigate short-term (e.g., seasonal) shifts in composition of the diet by mobilizing energy stores within their tissues. However, sea cucumbers have no dedicated energy storage organs (like the pyloric caeca in sea stars), and would thus have to store nutrients in other body tissues (Lawrence 1976, Oudejans and Van der Sluis 1979). In Chapter 3, I found clear relationships between tissue ratios, total lipid concentrations, and FA composition of skin and viscera and those of gonads, suggesting skin and viscera are storing lipids and FA that are later mobilized for gonad development. Other sea cucumber species also store lipids in skin during low-food periods (David and MacDonald 2002).

Deleterious shifts in diet could also be mitigated by the "conveyer belt" method of egg development exhibited by *P. californicus*, in which eggs mature over the course of three years in gonad tubules (Smiley and Cloney 1985, Smiley 1988). The initial formation of egg cell walls (composed of phospholipids) in gonad tubules occurs in years 1 and 2, while energy reserves (triglycerides) for pre-feeding larval development are laid down in year 3. This development pattern is unique to this species of holothurians (Sewell et al. 1997), and may extend development of eggs to correspond with seasonally available food supplies, limiting the need to mobilize lipids and FA from tissues.

I recommend further controlled feeding experiments that remove specific FA from diet to examine the effects of FA changes in food resources on multiple taxa. These experiments should

include multiple life stages (i.e., larvae, juveniles, and adults). In addition, longer-term (at least three years) studies should be conducted to cover the full length of egg development in species with "conveyer belt" egg production. Additional field surveys in the wild should also occur in multiple populations of *P. californicus*, specifically including their furthest distribution ranges in Kodiak, AK as well as Baja California, Mexico. By expanding the distribution and length of such studies, researchers would be better able to predict how populations of *P. californicus*, and potentially other deposit feeders, may react to changes in food supplies with climate change. My data have already been shared with researchers and industry personnel throughout Alaska and Washington State to support stock enhancement programs for populations that appear to be in decline due to fishing pressures (Bell et al. 2008, Clark et al. 2009), sea otter foraging (Kvitek et al. 1992, Larson et al. 2013), and possibly effects of climate-driven changes in food resources on female reproductive fitness.

By contributing research that is most needed for aquaculture and fisheries management, I have highlighted importance of applied ecological research in a broader sense where stakeholders are involved from the beginning of a study design. Although my work has made several contributions to improve current ecological knowledge, particularly for the Southeast AK population, there are still large gaps in the understanding of *P. californicus* life history, including natural recruitment, mortality, and predation rates. These data gaps, including identifying characteristics of ideal "nursery" areas (i.e., substrate, food, temperature) for *P. californicus* juveniles, will need to be filled if aquaculture production and stock enhancement of *P. californicus* is to continue successfully.

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Appendix A. Calibration curve equations used in all FA analysis

Table A-1. Calibration curve equations for the FAME external standard mixture

Fatty Acid	Calibration Equation	R^2
8:0	mg = 0.0037(peak area) - 0.0049	0.9885
10:0	mg = 0.0038(peak area) - 0.0105	0.9871
11:0	mg = 0.0038(peak area) - 0.0045	0.9867
12:0	mg = 0.0038(peak area) - 0.0096	0.9864
13:0	mg = 0.0038(peak area) - 0.0041	0.9861
14:0	mg = 0.0038(peak area) - 0.0100	0.9861
14:1ω5	mg = 0.0038(peak area) - 0.0057	0.9862
15:0	mg = 0.0038(peak area) - 0.0060	0.9863
15:1	mg = 0.0038(peak area) - 0.0056	0.9862
16:0	mg = 0.0038(peak area) - 0.0207	0.9865
16:1ω7	mg = 0.0043(peak area) - 0.0873	0.9979
17:0	mg = 0.0038(peak area) - 0.0075	0.9864
17:1ω9	mg = 0.0038(peak area) - 0.0058	0.9859
16:4ω1	mg = 0.0036(peak area) - 0.0008	0.9994
18:0	mg = 0.0038(peak area) - 0.0138	0.9862
18:1ω9 *trans*	mg = 0.0036(peak area) - 0.0052	0.9856
18:1ω9 *cis*	mg = 0.0036(peak area) - 0.0115	0.9860
19:0	mg = 0.0036(peak area) + 0.0101	0.9972
18:2ω6 *trans*	mg = 0.0038(peak area) - 0.0077	0.9861
18:2ω6 *cis*	mg = 0.0038(peak area) - 0.0115	0.9860
18:3ω6	mg = 0.0039(peak area) - 0.125	0.9867
18:3ω3	mg = 0.0038(peak area) - 0.0117	0.9870
18:4ω3	mg = 0.0034(peak area) - 0.0120	0.9869
20:0	mg = 0.0038(peak area) - 0.0088	0.9855
20:1ω9	mg = 0.0038(peak area) - 0.0056	0.9857
20:2ω6	mg = 0.0038(peak area) - 0.0089	0.9861
21:0	mg = 0.0038(peak area) - 0.0050	0.9858
20:3ω6	mg = 0.0078(peak area) - 0.0227	0.9868
20:4ω6	mg = 0.0097(peak area) - 0.0323	0.9870
20:3ω3	mg = 0.0023(peak area) + 0.1383	0.9932
20:5ω3	mg = 0.0026(peak area) + 0.1518	0.9957
22:0	mg = 0.0038(peak area) - 0.0080	0.9854
22:1ω9	mg = 0.0038(peak area) - 0.0051	0.9853
22:2ω6	mg = 0.0038(peak area) - 0.0075	0.9858
23:0	mg = 0.0038(peak area) - 0.0054	0.9818
24:0	mg = 0.0037(peak area) - 0.0049	0.9857
22:6ω3	mg = 0.0037(peak area) - 0.0208	0.9897
24:1ω9	mg = 0.0038(peak area) - 0.0038	0.9862

Appendix B. Data presented in Figure 2.6.

Table B-1. Mean values of standardized FA data for algal feed treatments, spawned gonads, and eggs for TW feed treatments.

FA	Feed Treatment	Spawned Gonad	Egg
12:0	0.0292	0.0112	10.2029
14:0	6.3658	0.9658	6.5482
14:1ω7	0.2103	0.0264	0.1516
14:1ω5	1.6312	0.1689	0.0541
14:1ω3	0.0000	0.9367	2.4607
15:0	4.9251	0.1083	0.5821
15:1	1.2380	0.1749	0.2025
16:0	20.3317	4.2011	27.2499
16:1ω13	0.0000	0.4584	0.4387
16:1ω11	1.4194	0.2251	0.2942
16:1ω9	0.0000	0.1244	0.3496
16:1ω7	21.2207	6.1638	3.2016
16:1ω5	0.3635	0.5152	0.1800
17:0	0.4813	0.8558	1.4208
16:3ω4	7.3434	0.1924	0.4343
17:1ω9	0.3474	0.0870	0.0290
16:4ω1	1.1423	3.0231	0.1414
18:0	0.7002	4.5881	20.2273
18:1ω13	0.0000	0.0928	0.0000
18:1ω9 *trans*	0.0000	0.1203	0.0959
18:1ω11	0.0000	0.0000	0.0000
18:1ω9 *cis*	0.2124	1.3262	1.8080
18:1ω7	0.2282	3.0587	1.9225
18:3ω6	0.0000	0.2674	0.0141
18:3ω4	0.0000	0.0289	0.0579
18:3ω3	0.2914	0.0469	0.6190
18:4ω3	0.8173	0.0000	0.0000
20:1ω11	0.0000	2.8820	0.9598
20:1ω9	0.0000	0.9251	1.0223
20:1ω7	0.0000	0.4963	0.3168
20:2ω9	0.0000	0.0000	0.0000
20:2ω6	0.0000	0.9327	0.0459
20:3ω6	0.0000	2.0759	1.1636

Table B-1 cont.

FA	Feed Treatment	Spawned Gonad	Egg
20:4ω6	1.4212	24.3470	0.6321
20:4ω3	0.0000	0.1826	0.0000
20:3ω3	0.8833	3.3337	1.4830
20:5ω3	14.7337	10.6240	2.0779
22:1ω9	0.0000	0.4445	0.7182
22:1ω7	0.0000	2.0512	0.9207
22:2ω6	0.7296	0.4396	0.0000
22:4ω6	0.0000	4.2414	0.0000
22:1ω1	0.0000	0.4660	0.0000
24:0	0.0000	0.4749	2.3524
22:6ω3	1.8818	11.3490	0.3663
24:1ω9	3.1883	1.5825	1.0012

Table B-2. Mean values of standardized FA data for algal feed treatments, spawned gonads, and eggs for TS feed treatments.

FA	Feed Treatment	Spawned Gonad	Egg
12:0	0.0000	0.0168	9.7400
14:0	0.3911	1.1493	6.7967
14:1ω7	0.2626	0.0614	0.0000
14:1ω5	1.9650	0.1372	0.0000
14:1ω3	0.0000	0.7763	3.5285
15:0	0.0520	0.1710	0.3560
15:1	1.3167	0.2489	0.0000
16:0	18.1730	4.7308	33.1284
16:1ω13	0.0000	0.2927	0.0000
16:1ω11	2.5755	1.0990	0.0000
16:1ω9	0.1410	0.8904	0.0000
16:1ω7	1.1347	3.8655	0.0066
16:1ω5	0.7718	0.8388	0.0000
17:0	0.0254	1.0341	1.0786
16:3ω4	0.0000	0.0689	0.0000
17:1ω9	1.4735	0.0713	0.0000
16:4ω1	1.2247	2.4099	0.0000
18:0	0.6328	4.4135	24.0603
18:1ω13	0.0000	0.1435	0.0000
18:1ω9 *trans*	0.0000	0.2193	0.0000
18:1ω11	0.0000	0.2624	0.0000
18:1ω9 *cis*	8.3011	2.3898	0.8392
18:1ω7	2.1624	3.0795	0.0000
18:3ω6	2.2213	0.7468	0.0000
18:3ω4	0.0000	0.0672	0.0000
18:3ω3	21.1742	0.2901	0.0000
18:4ω3	0.0000	0.0107	0.0000
20:1ω11	1.9065	3.4630	0.0000
20:1ω9	2.0153	1.5101	0.0000
20:1ω7	0.6852	0.6887	0.0000
20:2ω9	0.0000	0.0835	0.0000
20:2ω6	0.0000	1.0541	0.0000
20:3ω6	0.0000	1.6958	0.0000
20:4ω6	6.8801	22.9579	0.0000

Table B-2 cont.

FA	Feed Treatment	Spawned Gonad	Egg
20:4ω3	0.0000	0.9222	0.0000
20:3ω3	1.9254	0.8179	7.9019
20:5ω3	9.3670	5.3865	7.9019
22:1ω9	0.0000	0.9724	0.0000
22:1ω7	0.0000	1.8734	0.0000
22:2ω6	0.8572	0.4184	0.0000
22:4ω6	0.0000	5.7507	0.0000
22:1ω1	0.0000	0.9545	0.0000
24:0	0.0000	0.6708	0.0000
22:6ω3	0.0000	10.6047	0.0000
24:1ω9	0.0000	1.5664	0.0000